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CYANIDE HEMOCHROMOGEN

THE FERRIHEME HYDROXIDE-CYANIDE REACTION: ITS MECHANISM AND EQUILIBRIUM AS DETERMINED BY THE SPECTROPHOTOELECTRIC METHOD*

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The existence of a ferrihemochromogen[1] formed by a reaction between ferriheme and the cyanide ion has been known since 1871 when it was described by Preyer (2) as cyanhematin. The reversibility of this reaction was indicated in the studies of Anson and Mirsky (3), and of Hill (4) who studied analogous systems confined to the ferrohemochromogens, and by the work of Barron and Hastings (5) on potentiometric determinations with both the ferro and ferric forms of the cyanide compounds. This very probable reversibility led to our study of this equilibrium.

One purpose in making such an equilibrium study is to obtain information regarding the composition of both ferriheme and its cyanide derivative. Our work has led to the conclusion that ferriheme exists in solution as a polymerized ferriheme hydroxide (hematin) and that the cyanide compound is unpolymerized. Furthermore, this study demonstrates the advantages of an accurate spectrophotometric method in the study of equilibria involving the hemochromogens.

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[1] The cyanide derivative of hemin, ferriheme cyanide. The nomenclature was proposed by Pauling and Coryell (1).

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Ferriheme chloride (hemin) obtained by the usual preparative methods such as that of Willstätter and Fischer (6), and of Schalfejew (7) did not dissolve in alkaline solution to give good optical clarity. All commercial samples available likewise contained considerable quantities of cell material. Such materials were unfit for quantitative spectroscopic work. A better sample was prepared from dog blood (by E. S. G. B.) by an addition to the procedure of Willstätter and Fischer (6). The ferriheme chloride was dissolved in a mixture of quinine and chloroform and the solution was filtered through a Jena glass No. G4 filter. This solution was added dropwise to boiling acetic acid saturated with sodium chloride and the mixture left at room temperature for 24 hours; the resulting crystals of hemin were separated by filtration and washed with dilute acetic acid, water, alcohol, and ether. Two crystallizations followed this procedure, samples from which were dried at room temperature under a high vacuum at 10^{-5} mm. of Hg pressure. The absorption coefficients of both samples were identical within 1 per cent for all wave-lengths.

Analysis—3.83 mg.: 286.0 c.mm. N_2 (759 mm. Hg, 25°). Calculated, 287.6 c.mm. 8.12 mg.: 0.69 mg. Fe. Calculated, 0.696 mg.

This preparation dissolved completely and quickly in alkali stronger than pH 11. Upon dilution, stable solutions were obtained down to pH 6.44. More concentrated solutions could be obtained than with older preparations, and more reproducible oxidation-reduction potentials were also recorded (8).

Solutions of equilibrium mixtures were made by mixing stock solutions containing either sodium hydroxide alone or sodium hydroxide and potassium cyanide, with alkaline ferriheme solutions of known concentrations prepared from weighed samples. Proportions were varied by volumetric methods. In the experiments reported here the pH values were obtained by titration with standard HCl, the error being ±0.02 pH unit. No attempt was made to correct these values by a consideration of activity coefficients, for such corrections would make no appreciable difference. The 0.5 M cyanide stock solution was checked by titration with standard silver nitrate solution, the error being ±0.2 per cent. The total ferriheme concentrations were calculated from weight

and volumetric measurements which were accurate to ±0.5 per cent. This concentration was in all cases too low to necessitate correction of (OH⁻) or (CN⁻) for the amounts combined with the ferriheme. No precautions were taken for temperature control of the solutions, the room temperature being between 20–25°. Care was observed to prevent any appreciable absorption of atmospheric carbon dioxide. Equilibrium was established very rapidly.[2]

The absorption spectra were determined by the method described by Hogness, Zscheile, and Sidwell (9). The accuracy of this determination is indicated in the accompanying tabulation in which the comparison between our values for ferriheme hydroxide (hematin) and those of Drabkin and Austin (10) are given. It will be observed that the agreement is satisfactory within the limits of accuracy of the two determinations.

Wave-length........................	6150 Å. maximum	5700 Å. minimum
Drabkin and Austin, $\epsilon \times 1000$....... ...	4000–4500	3600–4000
Our values of $\frac{1}{2}$ mole $\alpha = \epsilon$...............	4590 ± 45	3650 ± 40

The ultraviolet and visible spectra of ferriheme hydroxide, $Hm_2(OH)_2$, in 0.1 M NaOH solution, and of ferriheme cyanide ion, $Hm(CN)_2^-$, in a solution containing 0.1 M NaOH and 0.5 M KCN per liter are shown in Fig. 1. These formulæ are the result of the work reported here. In this figure the absorption curves are plotted on the molecular basis. The spectroscopic experimental conditions at maxima and minima are given in Tables I and II.

For analysis of mixtures containing unknown proportions of 2 molecules of unknown formulæ, it is obvious that only the specific absorption coefficients can be used. Throughout this analysis the assumption is made that the specific absorption coefficient of the ferriheme radical in alkaline solution calculated on the ferriheme chloride weight basis remains constant even if associated.

[2] Experiments with phosphate buffers of various strengths showed that ferriheme combines with phosphate ions, a reaction which would unduly complicate the equilibrium system. Further work with these buffers was therefore discontinued. The phosphate compound has an absorption spectrum very much like that of the ferriheme hydroxide.
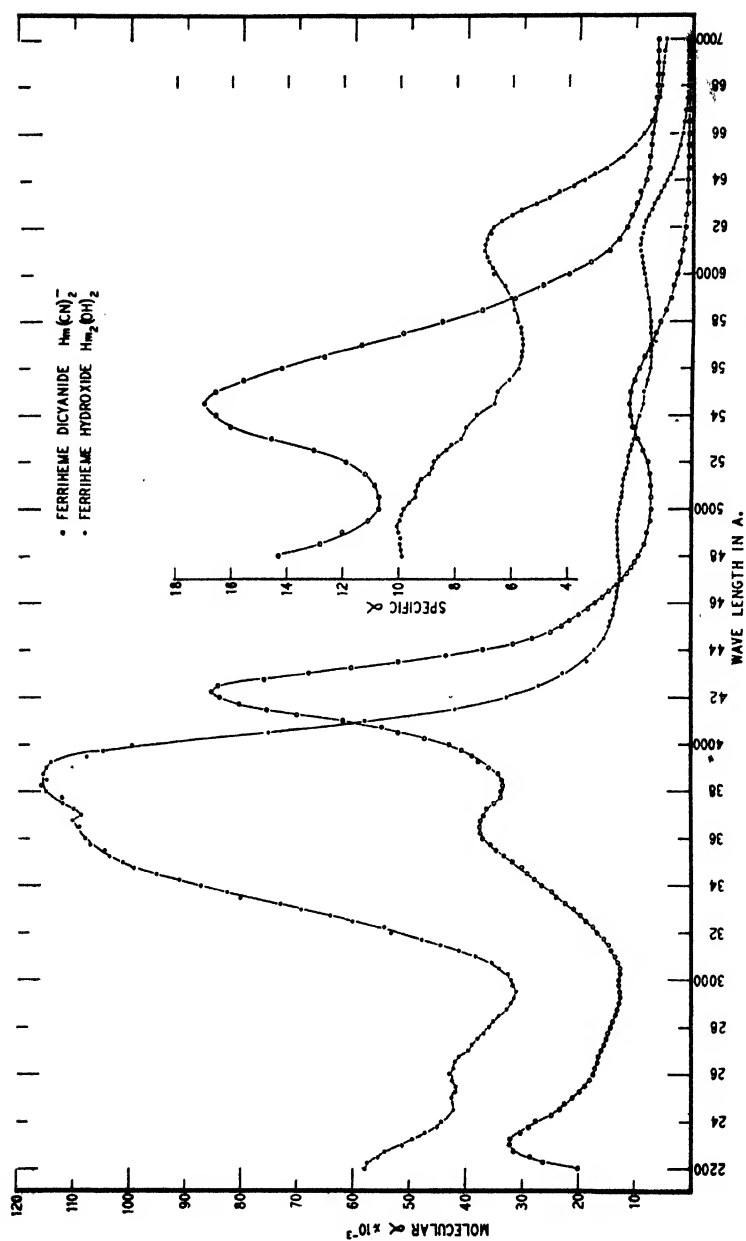
FIG. 1. Absorption spectra of ferriheme hydroxide and ferriheme dicyanide in 0.1 M NaOH solution. KCN 0.5 M in the case of ferriheme cyanide.

A portion of the spectrum is plotted on an enlarged scale on the specific basis. The wave-length 5450 Å. was used for analysis

TABLE I

Absorption Data for Ferriheme Hydroxide in 0.093 м NaOH Solution

Sample weight, 7 mg. of ferriheme chloride.   Cell thickness, 2 cm.

	Molecular $\alpha \times 10^{-3}$	Maximum deviation $(\pm)$ (2 runs)	Concentration	Slits	Spectral region isolated
			gm. per l.	*mm.*	*Å.*
Maxima ± 15 Å.					
2200	57.8	1.5	0.00328	0.25	10
2600	42.8	0.4	0.0052	0.07	6.3
3650	110.0	1.0	0.0019	0.05	16.2
3850	115.0	1.1	0.0019	0.07	26
4925	13.1	0.14	0.0158	0.009	2.1
6100	9.18	0.09	0.0241	0.005	2.4
Minima ± 15 Å.					
2950	31.1	0.3	0.0052	0.05	7.5
3700	108.8	1.0	0.0019	0.05	16.5
4750	12.7	1.5	0.0158	0.01	2.0
5700	7.3	0.08	0.0268	0.005	1.9

TABLE II

Absorption Data for Ferriheme Cyanide in a Solution Containing 0.093 м NaOH and 0.5 м KCN per Liter

Sample weight, 7 mg. of ferriheme chloride.   Cell thickness, 2 cm.

	Molecular $\alpha \times 10^{-3}$	Maximum deviation $(\pm)$ (2 runs)	Concentration	Slits	Spectral region isolated
			gm. per l.	*mm.*	*Å.*
Maxima ± 15 Å.					
2300	32.1	0.8	0.00297	0.3	18
3650	37.5	0.5	0.00297	0.07	22
4225	85.5	0.8	0.00113	0.03	3.6
5450	11.0	0.1	0.0096	0.01	3.0
Minima ± 15 Å.					
3000	12.7	0.2	0.00739	0.05	8
3825	33.6	0.3	0.00297	0.07	26
5000	7.0	0.7	0.01400	0.01	2.4

of the distribution of total ferriheme between ferriheme hydroxide and ferriheme cyanide.   At this wave-length the ratio of specific

absorption coefficients is 2.56, which is favorable for accurate analysis without change of cell length (9). The spectral region isolated for analysis was 1.6 to 3.2 Å., obtained by the use of 0.005 to 0.01 mm. slits. Values of specific α for 5450 Å. are 6.6 and 16.9 for ferriheme hydroxide and ferriheme cyanide respectively, calculated on the ferriheme chloride weight basis.

The points on Fig. 2 are the results of an experiment in NaOH solution, in which total ferriheme was kept constant and cyanide concentration was varied. A typical sigmoid curve was obtained. Studies of solutions with other pH values resulted in similar curves with displacements depending on the pH.

It was noted early in this study that apparent deviations from Beer's law occurred when the total ferriheme was not kept constant. This led to the following theory of dissociation. The reaction could be considered in a generalized way, but for clarity we propose immediately the equation we have finally deduced, and show that this is in agreement with experiment.

Consider the following reaction:

$$Hm_2(OH)_2 + 4CN^- = 2Hm(CN)_2^- + 2OH^- \tag{1}$$
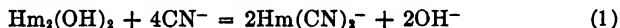
Hm represents the ferriheme radical. The equilibrium constant K for this reaction is

$$\frac{[Hm(CN)_2^-]^2(OH^-)^2}{[Hm_2(OH)_2](CN^-)^4} = K \tag{2}$$

or

$$\frac{[Hm(CN)_2^-](OH^-)}{\sqrt{[Hm_2(OH)_2]}(CN^-)^2} = \sqrt{K} = k \tag{3}$$

If Equation 3 be used as a guide for an experimental study, the following three experiments are suggested as tests of the mechanism of Equation 1.

Experiment A—Let (OH^-) and total ferriheme be constant.

$$\log \frac{[Hm(CN)_2^-]}{\sqrt{[Hm_2(OH)_2]}} = \log \frac{k}{(OH^-)} + 2 \log (CN^-)$$

$$\frac{d \log \dfrac{[Hm(CN)_2^-]}{\sqrt{[Hm_2(OH)_2]}}}{d \log (CN^-)} = 2$$

TOTAL (Hm)=8 x10⁻⁵ M
pH =13.24

% Hm(CN)₂⁻

(CN) x 10⁻³ M
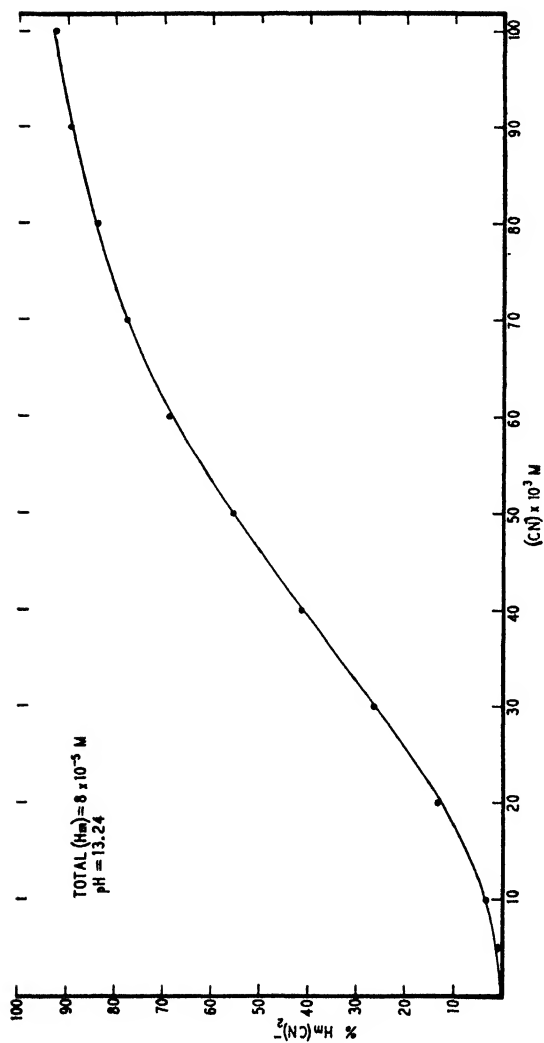
Fig. 2. Variation of composition with cyanide concentration

In Fig. 3 the values of log $\dfrac{[\text{Hm}(\text{CN})_2{}^-]}{\sqrt{[\text{Hm}_2(\text{OH})_2]}}$ are plotted as ordinates and the values of log (CN^-) as abscissæ. According to our interpretation the experimental values should fall on a straight line which has a slope equal to 2. In drawing these lines we have arbitrarily fixed the slope at a value of 2 and fitted a line of this
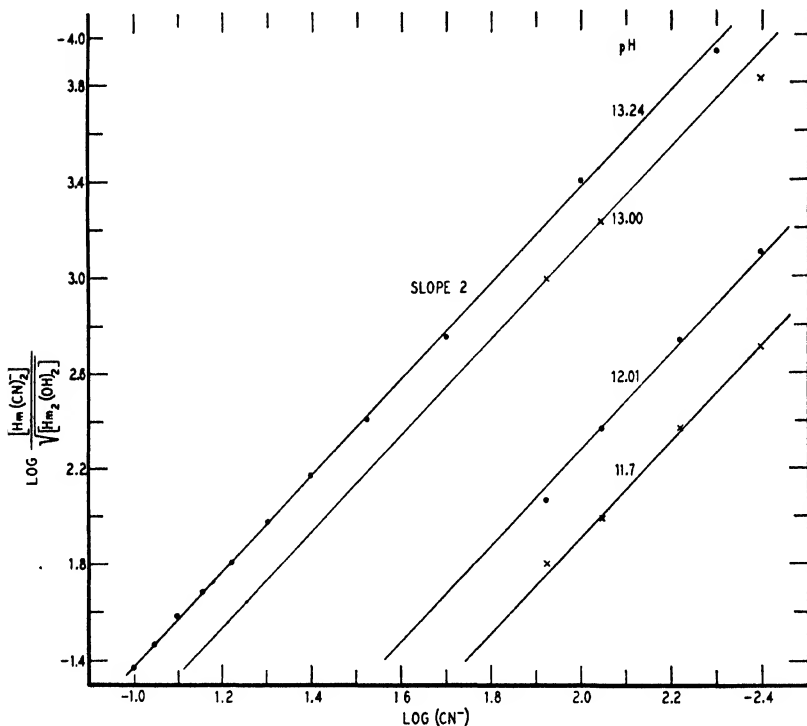
FIG. 3. Ferriheme hydroxide-cyanide equilibrium with solutions of different cyanide concentrations.

slope to the experimental values represented by the points on the plot. The agreement between experiment and theoretical interpretation is good for all pH values used. The data from which the plots were made are given in Table III.

The values of k calculated from the experimental curves are as follows:

pH	13.24	13.00	12.01	11.70
k	0.73	0.73	0.57	0.60

The values of k for the different pH values are not very concordant. However, the agreement is satisfactory when one considers that there was no thermostatic control and that the different determinations were made on different days. Since those solutions with the higher values of pH had optical properties which per-

TABLE III

Ferriheme Hydroxide-Cyanide Equilibrium with Solutions of Different Cyanide Concentrations

Total $(Hm) = 8.0 \times 10^{-5}$ M.

pH	(CN^-)	$Hm(CN)_2^-$ by weight on ferriheme basis	pH	(CN^-)	$Hm(CN)_2^-$ by weight on ferriheme basis
	$\times 10^3$ M	per cent		$\times 10^3$ M	per cent
13.24	5	0.93	13.00	4	1.2
	10	3.1		9	4.6
	20	13.2		12	7.9
	30	26.4	12.01	4	6.07
	40	41.1		6	13.6
	50	55.6		9	29.0
	60	69.1		12	49.0
	70	77.7	11.7	4	14.5
	80	83.8		6	28.6
	90	89.1		9	55.0
	100	92.6		12	69.5

mitted a wider range of study, the data on these solutions are regarded as the more reliable. With the value of k as 0.73, K has the value 0.53. The line through the experimental points in Fig. 2 is the result of a calculation using 0.53 for K in Equation 2.

Experiment B—Let (CN^-) and total ferriheme be constant. Then

$$\frac{d \log \dfrac{[Hm(CN)_2^-]}{\sqrt{[Hm_2(OH)_2]}}}{d \log (OH^-)} = -1$$

In Fig. 4 the appropriate values in the above expression are plotted and again the slope of the line is that predicted by the theory.   In these experiments the pH was varied while the cyanide and *total* ferriheme concentrations were kept constant.   The value of k calculated from the data in Table IV is 0.83.
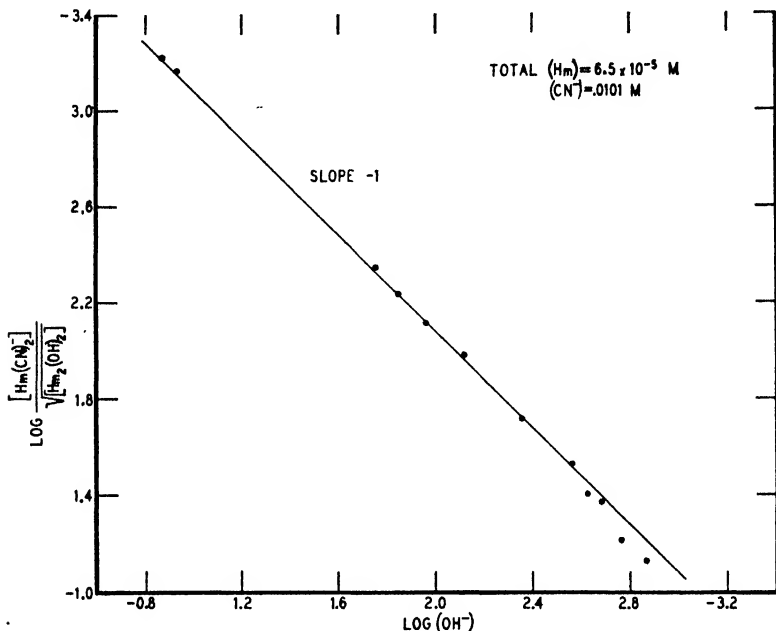
Fɪɢ. 4. Ferriheme hydroxide-cyanide equilibrium with solutions of different pH values.

Experiment C—Let (CN⁻) and (OH⁻) be constant.   Then

$$\frac{d \log [\mathrm{Hm(CN)_2^-}]}{d \log \sqrt{[\mathrm{Hm_2(OH)_2}]}} = 1$$

The experimental test of this proposition is shown in Fig. 5 and Table V.   The line has a slope of 1 and the points are experimental determinations at various concentrations of total ferriheme.   The value of k calculated from this curve is 0.72.   The time required for obtaining these data having been longer than for the tests on the propositions in Experiments A and B, there was greater temperature variation.   Furthermore, the analyses for the points at

the lower end of the line in Fig. 5 (lines through points) were less accurate than the others and less weight should therefore be attached to them. Again the agreement between theory and experiment is good. The data in Table V and Fig. 5 represent the crucial test for the dissociation of the molecule $Hm_2(OH)_2$. If

(CN⁻) omitted
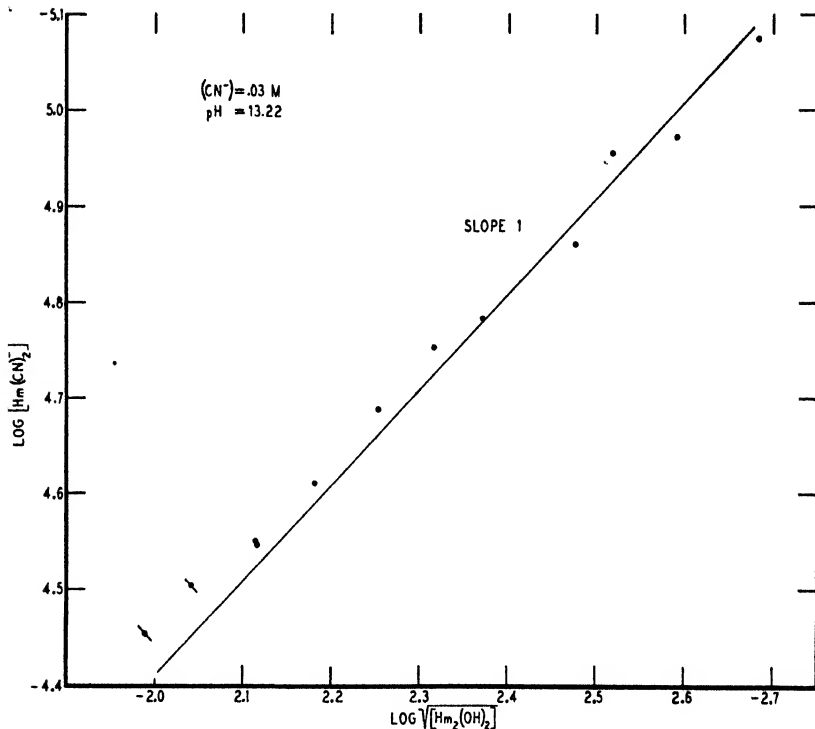
FIG. 5. Ferriheme hydroxide-cyanide equilibrium with solutions of different total ferriheme concentrations.

no dissociation had taken place, the fractional amount of the ferriheme hydroxide "cyanized" should have remained constant for all values of the total ferrihemochromogen concentration present in the solution.

Determinations made in solutions of high chloride ion concentration showed that this ion had no appreciable influence on the equilibrium. From this fact we conclude that ferriheme chloride in solution hydrolyzes to a very large extent to form the ferriheme

TABLE IV

Ferriheme Hydroxide-Cyanide Equilibrium with Solutions of Different pH Values

Total (Hm) = 6.5×10^{-5} M; (CN⁻) = 0.0101 M.

(OH⁻)	Hm(CN)₂⁻ by weight on ferriheme basis
$\times 10^{-5}$ M	*per cent*
136	5.15
117	5.91
17.6	32.7
14.3	39.5
11.0	48.5
7.70	59.0
4.38	78.5
2.71	88.6
2.38	92.9
2.05	93.9
1.72	96.7
1.39	97.8

TABLE V

Ferriheme Hydroxide-Cyanide Equilibrium with Solutions of Different Total Ferriheme Concentrations

(CN⁻) = 0.03 M; pH = 13.22.

Total (Hm) on ferriheme chloride weight basis	Hm(CN)₂⁻ by weight on ferriheme basis
$\times 10^{-5}$ M	*per cent*
24.0	14.7
19.6	16.0
14.6	19.4
14.6	19.3
11.1	22.0
8.3	24.7
6.42	27.6
5.25	31.5
3.7	37.4
2.95	37.7
2.40	44.6
1.70	49.6

hydroxide. Although hydroxide ion, like chloride ion, combines with ferriheme in the ratio of 1:1, cyanide ion combines in the ratio of 2:1. The existence of this ratio of two nitrogenous compounds

per heme seems to be a general rule in the formation of hemo-chromogens.   By chemical analysis this ratio of 2 has been found by Fischer, Treibs, and Zeile (11) for the pyridine, and by Langen-beck (12) for the imidazole and pilocarpine ferrihemochromogens; and by Zeynek (13) for the pyridine ferrohemochromogen.   By spectroscopic analysis, the same ratio has been reported for the ferroheme dicyanide by Anson and Mirsky (3) and for the nicotine and pyridine compounds by Hill (4).

To the best of our knowledge this work constitutes the first evidence of reversible polymerization of a hemochromogen.   On the basis of the structure as determined by Fischer and Zeile (14), the linkage between 2 such molecules in the formation of the dimer is not an obvious one.   An iron to iron atom linkage
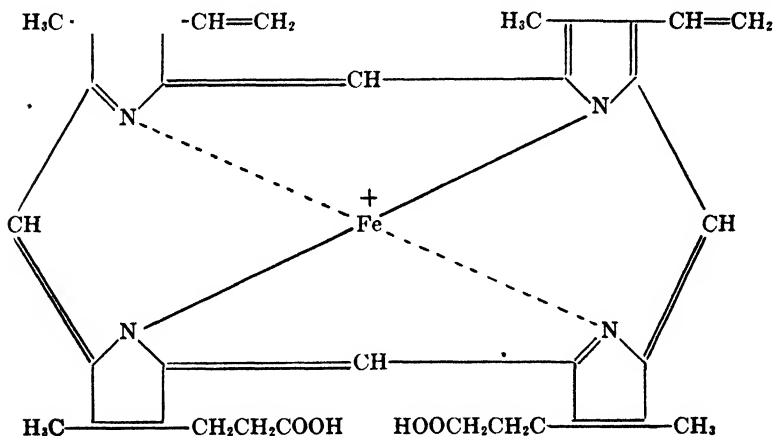
would satisfy coordination requirements, but no such linkages are known to us.   Furthermore, steric factors would very probably prevent the formation of a bond of this kind.   A completely satis-factory structure for this compound must account for this behavior.

SUMMARY

The equilibrium constant for the ferriheme hydroxide-cyanide equilibrium has been determined from spectrophotoelectric analy-ses made on equilibrium mixtures of ferriheme hydroxide and fer-riheme cyanide in the presence of cyanide in alkaline solution.   I

is shown that ferriheme hydroxide is associated, and that it dissociates upon reaction with cyanide, each ferriheme combining with 2 cyanide ions according to the following equation

$$Hm_2(OH)_2 + 4CN^- \rightleftharpoons 2Hm(CN)_2^- + 2OH^-$$
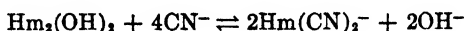
There is no apparent structural explanation for the associated compound.

Absorption spectra of ferriheme cyanide and hydroxide from 2200 to 7000 Å. are given.

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THE LIPID AND MINERAL DISTRIBUTION IN THE SERUM AND ERYTHROCYTES OF NORMAL CHILDREN

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The recent physiological and psychological studies, in all their many ramifications in the field of child development, emphasize anew the need for more complete knowledge of the intricate physiological changes that occur in the human organism accompanying its advances from infancy, through childhood, into the integrated state of the normal adult (1, 2).

It is well known that the human infant at birth is viable but not complete in structure and function, nor is it physiologically organized. It must pass through a series of changes before it reaches the steady functioning state characteristic of maturity. Frank (3) aptly states, "development implies concomitant changes in structures and functions. The fluctuations or oscillations in magnitude, *e.g.*, in intensity or duration or extension, which take place during growth may be regarded as essential in the growth process, since any fixity of function or process or of structure would interfere with, if it did not frustrate, growth changes. Growth and stability are not compatible, since organs must continue to function while growing."

To give an analogy in the simplest terms with regard to the growing child, it is like contending with problems involved by living in a house while one is building it. It is important to find a measure of the various stages of progress attained in logical sequence from the beginning to completion of the structure, upon which are superimposed the vital functions. For example, the number and structure of the specific blood cells may be taken not only as an index of the status of the tissues from which they are

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derived but of structural and functional changes (4), and in case of a pathological disturbance within the organism, they may reflect alteration in the fluid matrix and other tissues of the body. There are internal compensations taking place as growth proceeds, as is illustrated in the hematopoietic system, such as fluctuations and changes in configuration of the white blood cell count (5) and variations in the hemoglobin and erythrocyte content of the blood from infancy to maturity (6). Further, it has been shown that the relation of free and ester cholesterol in the blood, which appears to be a physiological constant in healthy adults and children, is not only different but more variable in the neonatal period of normal infancy and may be markedly changed in certain pathological conditions (7).

This investigation is one of several on the chemicophysiological changes coincident with growth and the transformation of the normal child from the period of variation and adjustment to that of mature stability. The present study which is concerned with the chemistry of the red blood cell and plasma in healthy growing children will indicate the magnitude of variability or fluctuations among individuals within a limited age range and also give a measure of the ability of the organism to maintain normal function in the face of changing structure (growth) and environment (food and living conditions). The hematological characteristics of the blood and certain of its constituents may, in addition, serve as indicators of the stage of physiological development which the individual has attained, as well as criteria of his state of health.

Accordingly the minerals, sodium, potassium, and chloride, and the various lipids have been determined simultaneously in the serum or plasma and the erythrocytes, accompanied by complete hematological observations of the blood, as well as certain physical studies of the erythrocytes such as cell volume, weight, diameter, thickness, specific gravity, water content, and resistance to hemolysis against saponin and hypotonic sodium chloride solutions.

Subjects—In an effort to obtain blood representative of the average normal preadolescent, twenty-six samples were secured from twenty-one normal children. The subjects have been divided into three groups, according to their nutritional stability, medical history, and environment. Groups 1 and 2 consist of sixteen children between the ages of 5 and 9 years who lived in the

Children's Village, an urban community with optimal environmental conditions such as sunshine, fresh air, and living arrangements simulating the average family unit.

Those in Group 1 had been especially selected for an extensive and continuous metabolic balance study in which the storage and interrelationship of nine different chemical elements had been observed over 8 consecutive months as growth and development had progressed.   They had been chosen because of a known clean health record and no detectable hereditary defects.   The excellence of their nutritive state was confirmed by careful medical examinations and periodic comprehensive anthropometric measurements[1] together with a detailed study of the bone development.[2]   In addition the children were of average to superior intelligence, emotionally stable, and good eaters with no known idiosyncrasies.   At the time the blood samples were taken these children were as well standardized as is possible in human subjects, because they had been on a controlled and fixed dietary regimen for the preceding 8 months during which time they had made excellent progress in growth and development.

The children in Group 2 had approximately the same environment as Group 1, with the exception that their diet was not controlled and they did not receive the close supervision demanded by a metabolic study.   Their bone development and health record were average, but more variable than those of the controlled group.

Group 3 consisted of five children, between the ages of 5 months to 5 years, from the Children's Hospital of Michigan.   These children had received treatment for various disorders such as burns, dislocations, and infections, but were considered normal from a clinical and hematological standpoint at the time the blood samples were taken.   However, in view of their past medical history with respect to infection and the possibility of suboptimal nutrition, inasmuch as they were of an indigent class and lower age range, they have been considered independently of Groups 1 and 2.

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[1] The medical examinations and anthropometric measurements of the children in Groups 1 and 2 were kindly made by Dr. Marsh Poole.

[2] A detailed study of the bone development was generously made by Dr. T. Wingate Todd and Dr. C. C. Frances of the Brush Foundation, Western Reserve University, Cleveland.

Methods

Blood Sampling—Postabsorptive blood samples were drawn from the arm vein. Part of the blood was immediately transferred from the syringe (without contact with the air) under paraffin oil to carry out anaerobic serum separation; the remainder was heparinized to prevent clotting.

Chemical Methods—Sodium was determined by the uranyl microgravimetric method ((8) p. 736); potassium by the cobaltinitrite microtitration method ((8) p. 748); and chloride by the open Carius method as applied by Van Slyke and Sendroy ((8) p. 836). The values for the mineral content of the cells were calculated from the determinations made on the whole blood and the serum, by the following formula.

$$C_c = \frac{C_b - C_s(1 - V)}{V}$$

where V is the hematocrit value of the percentage of red cells in 100 cc. of whole blood, and C_c, C_b, and C_s represent the concentrations in the cells, whole blood, and serum respectively.

Nitrogen was determined by the micro-Kjeldahl gasometric technique of Van Slyke ((8) p. 353; (9)). The lipids were determined by the gasometric procedure of Kirk, Page, and Van Slyke (10).[3, 4]

Different methods from those outlined by Van Slyke were used for the separation of the phospholipid into lecithin and cephalin.

[3] We wish to thank Dr. D. D. Van Slyke for so kindly accepting one of us (B. N. E.) in his laboratory to learn the gasometric lipid methods, and for his ready and helpful suggestions when advice has been sought concerning problems arising in the course of the study.

[4] Precautions were taken to extract the dried alcohol-ether residue immediately with petroleum ether. Experimental studies in this laboratory have shown that the phospholipids extracted by petroleum ether decrease if the dried alcohol-ether residue stands even overnight. Further work is being done upon variations in recovery of phospholipids, depending on the conditions of evaporation. It was found necessary to allow the strychnine ammonium molybdate reagent to stand for at least 2 weeks before it could be used for determination of the phosphorus. Experiments in this laboratory have shown that the solubility of the strychnine phosphomolybdate precipitate in acetone gradually increases until complete recovery of the phosphorus is secured only after the precipitating reagent is at least 2 weeks old.

It became evident to us, and has been pointed out by Van Slyke *et al.* in a more recent publication (11), that the determination of the amino nitrogen of the cephalin cannot be used because of contaminating nitrogen compounds which release part of their nitrogen in the amino form. Therefore, the following procedure, based upon the relative solubilities of lecithin and cephalin in absolute alcohol, was adopted tentatively.[5]

Total phosphorus was determined in the petroleum ether extract according to the method of Kirk (12). Additional samples of the extract were allowed to evaporate at room temperature in small beakers. They were extracted with six 2 cc. portions of cold absolute alcohol and filtered through 5 cm. No. 43 Whatman filter paper into phosphorus digestion tubes. The alcohol was evaporated, and the digestion, precipitation, and combustion carried out exactly as for the total phosphorus. Duplicate determinations checked closely and were within the limits of error stipulated for the total petroleum ether phosphorus. By subtracting the alcohol-soluble phosphorus from the total petroleum ether phosphorus it can thus be divided into two fractions, the absolute alcohol-soluble and absolute alcohol-insoluble phosphorus.

Resistance to Hypotonic Sodium Chloride[6]—A series of tubes containing 1 cc. of 0.70 per cent to 0.10 per cent sodium chloride was set up in intervals of 0.02 per cent. The concentrations in each tube were secured by measuring out from a burette, graduated in 0.02 cc., 1 per cent sodium chloride and the corresponding amounts of water. In the first tube were 0.70 cc. of sodium chloride and 0.30 cc. of water; in the second 0.68 cc. of sodium chloride and 0.32 cc. of water; in the third 0.66 and 0.34 cc. of sodium chloride and water, respectively. Thus the

first tube contained 1 cc. of 0.70 per cent, the second 1 cc. of 0.68 per cent, and the third 1 cc. of 0.66 per cent sodium chloride respectively.

When the tubes reached the temperature of the thermostat, 37°, 1 drop of blood was added to each, and thereafter they were shaken every 15 minutes for a period of 2 hours, were centrifuged, and a record made of the concentrations at which there was just beginning hemolysis and at which hemolysis was complete. This procedure separates the most resistant and least resistant cells in terms of sodium chloride.

Resistance to Saponin—The method of making the saponin dilutions outlined by Ponder ((13) p. 139) was modified to provide a more simple procedure and one in which the micrograms of saponin are calculated directly. The stock solution contains 125 mg. of saponin in 500 cc. of 0.9 per cent sodium chloride. Because of rapid deterioration it must be kept in the refrigerator and made fresh every 3 weeks. Dilute saponin solutions were made by measuring from a burette, graduated in 0.02 cc., the following amounts of stock saponin solution into 25 cc. volumetric flasks and diluting to volume with 0.9 per cent sodium chloride.

Stock saponin (amount diluted to 25 cc. to prepare the dilute standards)	Dilute saponin (1 cc. equivalent to)
cc.	*micrograms*
10.0	100
9.0	90
7.0	70
6.8	68
5.0	50
Etc.	Etc.

A series of tubes containing 1 cc. of the dilute standards, usually from 6 to 70 micrograms of saponin, was set up in intervals of 2 micrograms between 6 and 20; 5 between 20 and 45; and 2 between 46 and 70 micrograms. These ranges usually were sufficient for the most abnormal, fragile, or resistant cells. The tubes were placed in a thermostat at 37°.

1 to 2 cc. of the whole blood were centrifuged and the red cells washed three times with 0.9 per cent sodium chloride. A suspension of the washed cells in 0.9 per cent sodium chloride was made, usually 50 cc., and 1 cc. of this suspension was used in

each saponin tube.   The tubes were shaken at each half hour for 5 hours, were then centrifuged, and the concentrations at which initial and complete hemolysis occurred were determined.

Water Content—Samples of packed cells were weighed on a microbalance in small flat weighing bottles containing sand.   The water lost was calculated after the material was dried for 48 hours at 85°.

Specific Gravity—The specific gravity of the cells was calculated from the values of the plasma and whole blood, as determined by weighing in pycnometer bottles on the microbalance (14).

Cell Diameter—Bock's erythrocytometer, a simplified diffraction apparatus, was used to measure the average diameter directly from a dried smear of blood.   A recent excellent description of the instrument, technique, and precautions for its use is given by Sharp and Schleicher (15).

Corpuscular Measurements—Wintrobe's (16) methods were used in calculating cell volume from the percentage volume and red cell count of whole blood, and corpuscular hemoglobin from the red cell count and hemoglobin.   The weight was computed from the erythrocyte volume and specific gravity.   The thickness was determined from the cell volume and diameter by means of the three-dimensional chart adapted from von Boros by Haden (17), and volume thickness index, as outlined by Haden, with his nomogram adapted from Warburg.   Computations of corpuscular concentrations were made from the determined amount per 100 gm. of cells and the number of cells per unit weight, the latter value being calculated from the cell weight.

Hematological[7]—Red cell count, hemoglobin, white cell count, and differential were made on finger puncture blood.   Hemoglobin, red cell count, and cell measurements such as diameter and hematocrit were determined also in the heparinized venous blood in order to give values on cells for direct comparison with the chemical studies.   Pipettes certified by the United States Bureau of Standards were used for the red and white cell counts.   The Haden-Hauser clinical model, the accuracy of which was checked by Van Slyke's oxygen capacity method, was used for hemoglobin

[7] The blood samples were taken and many of the hematological observations were made by Dr. Pearl Lee.   We are indebted to her for selecting and making arrangements for the samples on the children in Group 3.

determinations. Differential white cell counts were made on films stained by Wright's technique.

DISCUSSION

Hematological—The hematological observations on the blood of the three groups of children are given in Table I. The individual red cell count and hemoglobin values fall well within the normal range published by Osgood and Baker for 215 children from 4 to 13 years of age (18). The narrow range of variation of red cell counts and hemoglobin furnishes further evidence of the normality of the children in the three groups with respect to any predisposition to anemia. The erythrocytes of the children in Groups 1 and 2, in accord with the observations of Osgood and Baker, appear to be smaller and contain less hemoglobin, thus accounting for lower hemoglobin values than are generally considered normal for adults. In this respect, however, the red cells of these children, particularly of Group 3, tend to be more like those of adults.

Minerals—Several studies dealing with the comparative sodium, potassium, and chloride content of serum and erythrocytes have been reported for adults (19–21). To our knowledge such a study has not been recorded for children.

Table II gives the results of the sodium, potassium, and chloride determinations in the red cells and serum of eighteen normal children (Groups 1 and 2) and the concentration of each mineral in the individual corpuscle. The average composition of the serum was found to be 142, 4, and 104 milli-equivalents of sodium, potassium, and chloride, respectively, per liter of serum. It is significant that the mineral composition of the serum is strikingly constant not only among the individual children of this study, but comparable to that reported for adults.

In contrast, however, there is a greater range of mineral values in the erythrocytes than in the serum. Although errors may be accumulated in calculating the normal composition of the erythrocyte from the serum and whole blood, it would seem that there are significant differences over and above any experimental error. The red cells of the children in Group 1, who had been maintained on a fixed dietary and metabolic regimen for 8 preceding months, showed a narrower range of variability in their potassium and

chloride composition than those of Group 2 who were living in the same environment but not controlled as to diet. Furthermore, the average potassium content of the erythrocytes in the former group appears to be significantly higher, whereas the average sodium and chloride concentrations are practically the same in the two groups. On the basis of both groups, the erythrocytes of normal children contain an average of 16, 115, and 53 milliequivalents of sodium, potassium, and chloride respectively, per liter of red cells.

In order to study the osmotic relationships between erythrocytes and plasma, the average results have been calculated in terms of milli-equivalents per liter of serum and cell water, as shown in parentheses in Table II. The averages for the normal children are 150, 4, and 110 milli-equivalents of sodium, potassium, and chloride, respectively, per liter of serum water. The values are much the same in the two groups and practically identical with those reported by Maizels on normal adults (21). Furthermore, for the children in Groups 1 and 2 and for adults, the average concentrations of chloride per liter of cell water are similar, with an average of 73 milli-equivalents. On the other hand, the concentration of potassium in cell water is different for Groups 1 and 2, with 169 and 154 milli-equivalents respectively, and is higher in comparison to 149 milli-equivalents for adults.

The sum of the total concentration of anions and cations in both erythrocytes and plasma has been calculated. The base combined with hemoglobin, on the basis of 45 milli-equivalents per liter of cells ((23) p. 99), and the values of Maizels (21) for bicarbonate in serum and cells have been used to calculate the additional ion constituents. An excess of approximately 16 ion milli-equivalents per liter of water was found in the serum over that in the cells for Group 2, which is comparable to an average of 21 milli-equivalents found for adults. The opposite was true, however, in the highly controlled group. Here the erythrocytes contained approximately 16 ion milli-equivalents in excess of that in the serum.

The average sodium, potassium, and chloride concentrations of a single erythrocyte are 14, 98, and 45×10^{-13} milli-equivalents respectively. The narrower range of variation and higher potassium and chloride contents of the average individual erythro-

TABLE I

Hematological Observations on Normal Children

Subject	Sex	Age		Red cells		Hemoglobin		Hematocrit	Corpuscular measurements						Hemolysis			
															Hypotonic NaCl		Saponin	
				Whole blood	Per gm. cells	Whole blood	Cells		Vol-ume	Weight	Diam-eter	Thick-ness	Diam-eter thickness ratio	Volume thickness index	Begin-ning	Com-plete	Begin-ning	Com-plete
		yrs.	mos.	millions	× 10¹⁰	gm. per 100 cc.	micro-micro grams	per cent	c.μ	micro-micro grams	μ	μ			per cent	per cent	micro-grams	micro-grams
Group 1																		
J. H.	M.	5	3	4.78	1.11	12	25	40	83	90	7.3	2.0	3.6	1.08				
B. M.	F.	5	11	4.76	1.03	12	25	42	88	97	7.2	2.2	3.3	1.21				
B. S.	M.	6	1	4.39	0.93	12	27	42	96	108	7.2	2.4	3.0	1.32				
B. F.	F.	6	3	4.55	1.04	12	26	40	88	96	7.2	2.2	3.3	1.21				
J. M.	"	6	9	4.81	1.07	12	25	42	86	94	7.3	2.1	3.5	1.12				
F. C.	M.	7	2	4.15	1.03	11	27	37	90	97	7.4	2.0	3.7	1.14				
H. H.	"	7	7	4.71	1.03	13	28	42	89	97	7.2	2.2	3.3	1.22				
D. P.	"	8	10	4.38	1.14	12	27	35	81	88	7.2	2.0	3.5	1.11				
Average				4.57	1.05	12	26	40	88	96	7.3	2.1	3.4	1.17				

oup.

Subject	Sex																	
R.O.	M.	4	10	5.83	1.37	14	23	39	67	73	7.4	1.6	4.6	0.85	0.38	0.26	10	56
C.S.	"	8	1	5.39	1.12	13	24	44	82	89	7.1	2.2	3.2	1.25	0.42	0.26		
"	"	8	5	5.21	1.14	15	29	46	88	96	7.5	1.8	4.2	0.98	0.36	0.24		
B.O.	"	6	4	5.07	1.15	13	25	41	81	88	7.4	2.2	3.4	1.17	0.38	0.24	10	58
F.C.	"	6	6	4.47	0.99	11	25	36	80	87	7.0	2.0	3.5	1.29	0.42	0.30	10	53
G.S.	"	7	3	4.23	1.06	12	27	39	93	101	7.1	2.1	3.4	1.14	0.40	0.26	10	52
"	"	7	5	4.72	1.15	14	30	41	87	95	7.0	2.0	3.5	1.11	0.42	0.28		
C.M.	"	7	6	5.12	1.22	14	27	41	80	87	7.2	2.1	3.4	1.15	0.42	0.26		
I.S.	F.	8	1	5.74	1.10	15	26	43	75	82	7.2	2.0	3.5	1.15	0.40	0.32		
D.P.	M.	8	2	4.40	1.09	12	26	37	84	91	7.1	2.2	3.2	1.24	0.40	0.26	9	50
P.O.	"	8	8	4.77	1.07	14	29	40	84	92	7.2	2.2	3.3	1.21	0.42	0.26		
"	"	9	1	4.48	1.04	13	29	39	87	94								
M.W.		8	11	4.74		15	32	42	88	96							10	40
Average				4.94	1.12	13	27	41	83	90	7.2	2.0	3.6	1.14	0.39	0.28	10	52
" for Groups 1 and 2				4.80	1.09	13	27	40	85	92	7.2	2.1	3.5	1.16			10	

Group 3

Subject	Sex																	
A.A.	M.	0	5	4.25	1.03	14	33	38	89	97					0.53	0.30	10	33
P.N.	F.	2	0	4.41	1.01	14	32	40	91	99					0.50	0.31	10	33
J.S.	M.	2	1	4.07	0.92	13	31	41	100	109					0.44	0.22	9	33
J.D.	F.	5	0	4.72	1.08	14	30	40	85	93					0.42	0.32	10	33
J.L.	M.	5	0	4.48	1.09	16	37	38	84	92					0.44	0.20	8	25
Average				4.39	1.03	14	32	39	90	98					0.47	0.27	10	32

TABLE II

Distribution of Sodium, Potassium, and Chloride in Blood of Normal Children

Subject	Sex	Age		Serum			Erythrocytes			Corpuscle[*]		
				Sodium	Potassium	Chloride	Sodium	Potassium	Chloride	Sodium	Potassium	Chloride
		yrs.	*mos.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	$\times 10^{-13}$ *m.-eq.*	$\times 10^{-13}$ *m.-eq.*	$\times 10^{-13}$ *m.-eq.*

Group 1

Subject	Sex	yrs.	mos.	Sodium	Potassium	Chloride	Sodium	Potassium	Chloride	Sodium	Potassium	Chloride
J. H.	M.	5	3	138	4	101	15	128	51	12	106	42
B. M.	F.	5	11	143	4	103	11	120	50	10	106	44
B. S.	M.	6	1	139	4	99	20	122	52	20	122	52
B. F.	F.	6	3	137	4	105	14	125	43	13	117	45
J. M.	"	6	9	139	4	110	17	128	66	15	110	57
F. C.	M.	7	2	135	4	103	27	112	59	24	100	48
H. H.	"	7	7	130	4	97	21	117	58	19	104	51
Average.............				137	4	103	18	122	54	16	109	48
				(146)	(4)	(108)	(25)	(169)	(75)	(22)	(152)	(67)

Group 2

Subject	Sex	yrs.	mos.	Sodium	Potassium	Chloride	Sodium	Potassium	Chloride	Sodium	Potassium	Chloride
R. O.	M.	4	10	145	4	105	16	112	48	10	75	32
C. S.	"	8	1	147	4	109	20	109	58	16	90	48
B. O.	"	6	4	146	4	108	16	102	45	13	82	37
F. C.	"	6	6	147	4	111	18	116	50	15	93	40
G. S.	"	7	3	147	4	106	4	115	53	4	107	49
C. M.	"	7	6	142	4	96	10	100	35	7	80	28
L. S.	F.	8	1	145	4	104	15	110	55	11	82	41
D. P.	M.	8	2	146	4	107	21	100	38	18	84	32
C. S.	F.	8	5	141	5	102	23	106	57	20	93	50
P. O.	M.	8	8	143	5	97	12	105	45	10	88	38
"	"	9	1	139	4	101	14	142	82	12	124	72
Average.............				144	4	104	15	111	51	12	91	42
				(153)	(4)	(111)	(21)	(154)	(72)	(18)	(126)	(59)
Total average.......				142	4	104	16	115	53	14	98	45
				(150)	(4)	(110)	(23)	(160)	(73)	(19)	(136)	(62)

The figures in parentheses represent average milli-equivalents per liter of serum and cell water, and $\times 10^{-13}$ per corpuscular water. Calculations are based on the average serum water of 94.4 per cent given by Peters and Van Slyke ((22) p. 753) and cell water of 66 per cent by weight or 72 per cent by volume as determined in these studies.

[*] Represents the concentration in an average single red blood cell.

cytes of the children on fixed dietary suggest that these constituents in the cell may be affected by diet (24). Since the serum appears to maintain a constant state in the mobilization of base, it seems that more attention should be given to the composition of erythrocytes in seeking indicators of stages in physiological development, growth, and state of health.

Lipids—There are numerous studies on the plasma lipids, but very few deal with the complete lipid distribution, particularly in children. Most of the studies include only one lipid constituent, such as the relation of the free and ester cholesterol in the extensive studies of Sperry (7, 25). As to observations of lipids in the red blood cell, few have been reported on humans and those have been on mature individuals.

The values of the blood lipids in normal children are presented in Table III. It can be seen that the concentration of the various lipid fractions in the plasma of the children in Groups 1 and 2 are similar, whereas those for Group 3 are higher. Two of the children in the hospital group show evidence of a slight lipemia (P.N. and J.S.). It was thought best, owing to their past history and uncertain normality, as well as to the smaller number and wider range in total lipid values than in the other groups, not to include them in the general average.

With the same method, a comparison of the values of normal children with the adult values of Page, Kirk, Lewis, Thompson, and Van Slyke (26) shows that all the various lipid fractions of the plasma are markedly lower in preadolescence in spite of considerable individual variations. The comparative values in mg. per 100 cc. are as follows: total lipids, adults 735 ± 216, children 454 with a range of 333 to 606; cholesterol esters, adults 254, children 182 with a range of 109 to 235; phospholipids, adults 181 ± 71, children 136 with a range of 52 to 175; neutral fat, adults 225 ± 137, children 100 with a range of 0 to 178; free cholesterol, adults 82 ± 17, children 35 with a range of 17 to 46. Values obtained by other methods (26) on normal adult plasma are in general intermediate between those of our children and the adults of Page *et al.* (26).

The Rockefeller group have studied the effect of advancing age from 20 to 100 years of age, but have found no difference in plasma lipids. However, the effect of age during the growing period,

TABLE III

Blood Lipids of Normal Children

Subject	Sex	Age (yrs)	Age (mos)	Plasma per 100 cc.						Erythrocytes per 100 gm.						Corpuscle*					
				Total lipid	Phospho-lipid	Neutral fat	Cholesterol Total	Free	Esters	Total lipid	Phospho-lipid	Neutral fat	Cholesterol Total	Free	Esters	Total lipid	Phospho-lipid	Neutral fat	Cholesterol Total	Free	Esters
				mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	$\times 10^{-12}$ mg.	$\times 10^{-12}$ mg.	$\times 10^{-12}$ mg.	$\times 10^{-12}$ mg.	$\times 10^{-12}$ mg.	$\times 10^{-12}$ mg.
J. H.	M.	5	3	434	142	66	152	45	182	448	271	56	115	104	18	404	244	50	104	94	16
B. M.	F.	5	11	388	113	93	119	28	154	404	233	30	124	99	42	392	226	29	120	97	41
B. S.	M.	6	1	358	118	114	82	17	109	421	247	41	110	77	55	455	267	44	119	83	59
B. F.	F.	6	3	495	109	178	140	41	167	340	191	34	112	108	8	349	196	35	115	111	8
J. M.	"	6	9	606	175	159	177	38	235	443	239	76	120	109	19	416	224	71	113	103	18
F. C.	M.	7	2	474	168	39	170	31	235	457	259	16	154	114	67	444	252	16	150	111	69
H. H.	"	7	7	462	158	70	152	34	200	435	256	78	101	99	3	421	248	76	98	96	3
D. P.	"	8	10	401	144	35	144	31	191	394	239	24	112	82	49	346	210	21	98	72	43
Average..........				452	141	94	142	33	184	418	241	44	119	99	34	403	233	43	115	96	32

Group 1

Group 2

	Sex	Yr.	Mo.																			
C. S.	M.	8	5	561	158	174	154	46	182	437	254	40	128	109	33	419	244	38	123	106	32
B. O.	"	6	6	488	138	137	143	41	173	415	198	117	100	100	0	420	232	63	114	97	27
G. S.	"	7	5	482	147	110	149	38	188	444	245	67	121	103	29	369	219	13	112	76	60
C. M.	"	7	6	373	52	139	120	31	150	425	253	15	129	88	69	375	217	60	88	73	17
I. S.	F.	8	1	521	156	159	139	41	165	459	266	73	108	89	32	427	225	104	97	96	2
P. O.	M.	8	8	428	154	0	180	45	229	467	246	114	106	105	2	360	225	22	97	74	38
M. W.	"	8	11	333	98	45	122	24	166	375	235	23	101	77	40						29
P. O.	"	9	1	459	149	76	152	34	200	416	278	13	112	94	31	391	261	12	105	88	
Average				456	132	105	145	37	183	430	247	58	113	96	29	394	232	45	105	87	30
" for Groups 1 and 2				454	136	100	143	35	182	424	244	51	116	97	32	399	233	44	110	92	30

Group 3

	Sex	Yr.	Mo.																			
A. A.	M.	0	0	518	140	178	141	56	144	403	218	25	147	128	32	403	211	24	142	124	31
P. N.	F.	2	0	755	201	170	260	79	305	434	273	16	145	145	0	430	271	16	144	144	0
J. S.	M.	2	1	804	247	307	172	59	191	453	254	69	130	130	0	491	275	75	138	138	0
J. D.	F.	5	0	619	210	112	203	67	230	423	232	21	160	146	23	394	216	20	149	137	21
J. L.	M.	5	0	371	87	141	100	38	105	495	288	62	133	115	30	453	264	57	122	105	27
Average				613	177	182	175	60	194	442	253	39	143	133	17	434	247	38	139	130	15

* Represents the concentration in an average single red blood cell.

when structural changes are in progress, may be quite different than after maturity, as indicated by the comparison of results on adults with children. In the erythrocytes, the average total lipid concentration is similar to that of the plasma; however, the distribution is decidedly different. Phospholipid and free cholesterol have the highest concentration, whereas in plasma, cholesterol esters and phospholipid are highest. Cholesterol esters are at a minimum in cells and sometimes entirely absent. We have found significant amounts to be present, however, in contrast to the general belief that there is little if any ester present in the cells. Free cholesterol has the lowest concentration of all the lipids in the plasma, but significant amounts are always present in the cells. The neutral fat is the most variable, both in plasma and cells, but appears to have approximately twice the concentration in plasma as occurs in the erythrocytes. Inasmuch as the neutral fat is a calculated value, less significance can be attached to its relative concentrations.

In the cells, the values for total lipid, phospholipid, and neutral fat are similar in all three groups. The total cholesterol is higher in Group 3 than in Groups 1 and 2 in spite of a decrease in the ester fraction, the increase being reflected by the elevation in the free cholesterol. For reasons stated previously, the less standardized Group 3 has not been included in the general average. The average lipid values in mg. per 100 gm. for the cells of eighteen children (Groups 1 and 2) are as follows: total lipid, 424; phospholipid, 244; free cholesterol, 97; neutral fat, 51; and cholesterol esters, 32. Since there are few normal values for the concentration of the lipids in the cells of humans in the literature, Dr. Donald D. Van Slyke has been kind enough to furnish us with some of his values obtained on thirteen adult men.[8] Using his analytical methods, we have found that the total lipids, neutral fat, and cholesterol esters are similar for the erythrocytes of growing and mature subjects. On the other hand the phospholipid concentration of the cells of normal children is higher and the free cholesterol is lower.

The percentage distribution of the various fractions in the total lipids of the blood and the percentage of free cholesterol in total

[8] Personal communication with Dr. D. D. Van Slyke.

cholesterol are given in Table IV. Although the absolute concentration of the different lipids may vary normally over a wide range, particularly in the plasma, the individual constituents change·concomitantly, thus maintaining a characteristically constant percentage distribution. It can be observed readily that

TABLE IV

Percentage Distribution of Lipids in Blood of Normal Children

	Plasma				Erythrocytes				Plasma	Erythrocytes
	Cholesterol esters	Phospholipids	Neutral fat	Free cholesterol	Cholesterol esters	Phospholipids	Neutral fat	Free cholesterol	Free in total cholesterol	
Group 1 (8 subjects)										
	per cent of total lipids	per cent of total lipids	per cent of total lipids	per cent of total lipids	per cent of total lipids	per cent of total lipids	per cent of total lipids	per cent of total lipids	per cent	per cent
Minimum..... ·........	30	22	8	5	1	54	3	18	18	70
Maximum..............	50	35	36	10	15	61	18	32	30	98
Average................	41	31	21	7	8	58	10	24	23	83
Group 2 (8)										
Minimum..............	32	14	0	7	0	48	3	19	20	68
Maximum..............	54	36	37	10	16	67	28	25	39	100
Average................	40	29	23	8	7	58	13	22	26	85
Total average for Groups 1 and 2.......	40	30	22	8	7	58	12	23	24	84
Group 3 (5)										
Minimum..............	24	23	18	7	0	54	4	23	33	83
Maximum..............	40	34	38	11	8	63	15	35	40	100
Average...............	31	29	30	10	4	57	9	30	34	93

the percentage distribution of the plasma lipids is practically identical in Groups 1 and 2; however, that of Group 3, although considered clinically normal from the hematological standpoint, is different. When compared with the data of Page *et al.* (26), where they demonstrate the change of plasma lipid composition with varying degrees of normal lipemia, the results of Group 3

agree closely with their values where the concentration of the total lipid lies between 651 and 800. Further, the percentage distribution in the plasma of the children in Groups 1 and 2 is similar to that of adults, where the concentration of total lipids ranges between 400 and 650 mg. per 100 cc. of plasma. The majority of our fifteen normal children in the standardized groups falls within this range in total lipid concentration; several fall below; but none rises above.

In the plasma, over two-thirds of the total lipid is made up of cholesterol esters and phospholipid, one-fifth by neutral fat, and less than 10 per cent by free cholesterol. In the cells a decidedly different picture exists. Here, approximately 80 per cent of the total lipid is phospholipid and free cholesterol. The remaining 20 per cent is divided between neutral fat and cholesterol esters in the ratio of about 2:1.

The recent studies of Sperry (7, 25) on the cholesterol content of plasma and serum in addition to establishing normal ranges of both free and combined cholesterol for the human infant and adults, have brought out these interesting facts: (1) new born infants have less cholesterol ester in the blood plasma and show a wider range of variation than adults; (2) the percentage of free in total cholesterol appears to be a physiological constant in normal blood serum or plasma. Sperry's results show that in children and adults the normal range of the percentage of free in total cholesterol is 24 to 31 per cent, with an average of 27 per cent. The data of Page et al. (26) demonstrate a range for normal adults between 22 and 72 per cent, with an average of 37 per cent. Our data on fifteen preadolescent children (5 to 9 years of age) indicate more variation than those of Sperry, but much less than those of Page et al. The results presented in Table IV show a range of 18 to 39 per cent, with an average of 24 per cent of free in total cholesterol, which is in close agreement with that of Sperry. The values for the children in Group 3, in which several of the children have evidence of lipemia, agree more closely with the values of Page et al. The range is from 23 to 40 per cent of free in total cholesterol with an average value of 34 per cent. The fact that the children in Group 3 were in a lower age range may offer a further explanation of the increased concentration of free in total cholesterol. A recent report by

Offenkrantz and Karshan (27) on the serum cholesterol of 250 children ranging in age from 2 months to 12 years shows that the free cholesterol was similar at all age levels, whereas the total cholesterol tended to be lower for the younger children. The children in the lowest age group, therefore, had a higher percentage of free in total cholesterol in the serum.

In the cells in which the major portion of the cholesterol is in the free state the values for children evidence a range of 68 to 100 per cent with an average of 84 per cent. Data from Van Slyke's laboratory on normal adults are similar.

In efforts to separate the phospholipids into individual fractions, we have found 91 per cent of the petroleum ether-soluble phosphorus of the plasma and 80 per cent of that in the erythrocytes soluble in cold absolute alcohol. The individual values of subjects in Groups 1 and 2, upon which these averages are based, were characteristically constant and suggest that the percentage distribution of the phospholipid into its separate fractions may prove to be as physiologically constant as the distribution of total lipids and cholesterol.

A consideration of the growth processes (changing structure and function) in the light of both minerals and lipids of the plasma, together with the hematological observations, offers evidence suggesting that the concentration of minerals may indicate the ability of the organism to maintain equilibrium in spite of changing structure and function, whereas the lipids may indicate the progressive stages of development between infancy and maturity. Certain characteristic differences of the plasma lipid found between the neonatal period, preadolescence (5 to 9 years), and maturity suggest a fruitful field of investigation in extending the present data and exploring other periods of advancement, such as infancy, early childhood, and puberty.

On the other hand, the greater constancy of lipids in the erythrocytes among individuals, as well as between children and adults, indicate that they are a more fundamental part of the structure itself and thus not as likely to be influenced by changes accompanying growth and environmental conditions. The minerals of the erythrocytes are probably more mobile constituents and may reflect the nutritional status and certain environmental influences, such as diet.

SUMMARY

Simultaneous analyses of the minerals and lipids in the plasma and cells of preadolescent children, together with complete hematological observations and certain physicochemical studies, have been made in order to determine not only the variations among normal children[9] but also possible indications of the various stages of development from the period of viability to maturity.

The results present evidence that the lipid concentration of the blood plasma in preadolescence is lower than in adults. The hematological studies give further evidence of the difference between maturity and the growth period. The minerals in the serum of normal children are strikingly constant, and similar to those reported for adults, whereas in the cell they exhibit more variability and appear to be more susceptible to environmental influence such as diet. The concentration of lipids in the cells of children is similar to that in adults and, being fundamental constituents of the cell structure, less likely to be influenced by the process of growth or changing environment.

The calculation of results on the basis of amounts per corpuscle rather than concentration per unit weight or volume of cells presents a different picture of the individual erythrocyte. The possibility of using certain of the blood constituents as indicators of the stage of development during growth has been discussed.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF SERUM MAGNESIUM BASED ON THE HYDROXYQUINOLINE PRECIPITATION*

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Since 1922, the most common method for the determination of serum magnesium has been its precipitation as $MgNH_4PO_4 \cdot 6H_2O$ and the determination of the phosphate in this precipitate colorimetrically (1). Though this method can be used successfully and has been the basis for most of our knowledge of blood magnesium concentrations, it has never been regarded as satisfactory. The difficulty of obtaining complete precipitation of the minute amount of $MgNH_4PO_4 \cdot 6H_2O$ from solutions with notorious tendencies to remain supersaturated, the uncertainty of the composition of the precipitate, the technical hazards of washing the precipitate in centrifuge tubes, the independent uncertainties of colorimetric phosphate procedures, as well as the length of time required for the analysis, have all combined to make investigators welcome the proposal of other methods.

Among other micromethods for magnesium, the one which has engaged the most attention has been that based on the precipitation of magnesium 8-hydroxyquinoline in hot ammoniacal solution. This principle was first utilized as the basis of a gravimetric method by Hahn (2) in 1926. Then Berg (3) adapted the principle to microtechnique by determining the hydroxyquinoline by bromination with KBr and standard $KBrO_3$ using indigo carmine as an indicator. The bromination technique was further modified by Bomskov (4) and by Greenberg and Mackey (5), who determined the excess bromate iodometrically. The latter authors

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* An abstract of this paper was presented before the Central Society for Clinical Research, November 6, 1936.

have further altered the method by forsaking the centrifuge tube, using instead the Kirk-Schmidt (6) microfilter, and by carrying on the titration in a special flask to avoid loss of bromine. Other modifications of the bromination technique have been adapted in France by Glomaud (7), Javillier and Lavollay (8), and Velluz (9).

The hydroxyquinoline has been determined also colorimetrically, as in the method of Yoshimatsu (10) and of Eichholtz and Berg (11), who used the Folin phenol reagent to produce a blue color.

These many attempts to utilize the principle of precipitating magnesium as magnesium 8-hydroxyquinoline—as well as many other methods not mentioned—testify that the principle involved is apparently an advantageous one, that could be perfected sufficiently to supplant the methods involving the precipitation of $MgNH_4PO_4 \cdot 6H_2O$. During the past year, the author has been attempting, too, to adapt one of the many modifications of the hydroxyquinoline methods into a workable and reliable procedure. He has, however, been forced to modify his technique many times before he finally arrived at one that would give consistently reliable results. In the course of this work, a new colorimetric method for the determination of 8-hydroxyquinoline was developed, which when applied to the determination of magnesium simplified and increased the accuracy of the determination. This colorimetric method is based on the fact that 8-hydroxyquinoline combines with ferric ions in weak HCl to form a specific green-blue color, which is easy to determine in a colorimeter, and which in a photoelectric colorimeter gives a straight line curve on semilogarithmic paper.[1]

Method

Reagents and Special Apparatus—

6 per cent hydroxyquinoline in alcohol. 8-Hydroxyquinoline is purified by recrystallizing twice from dilute ethyl alcohol. A

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[1] After the experimental work on this paper was completed, it was discovered that Lavollay (12), in a paper on the microdetermination of iron by precipitation with 8-hydroxyquinoline, suggested the use of the green-blue color formed when iron 8-hydroxyquinoline is dissolved in alcohol for the colorimetric determination of magnesium. He gave, however, no details of the method and no quantitative data.

small amount of a 6 per cent solution in 95 per cent ethyl alcohol is prepared and is kept in a dark bottle with a stopper containing an eye-dropper. The bottle should be stored in a dark place. A new solution should be prepared about once a month.

Saturated solution of magnesium hydroxyquinoline. About 500 cc. of a solution containing about 10 mg. of Mg per 100 cc. are treated with about 5 cc. of a saturated solution of NH_4Cl, then with about 5 cc. of 6 per cent hydroxyquinoline in alcohol. Concentrated ammonia is slowly added until the solution is strongly alkaline, and the material is heated to boiling. The greenish-yellow precipitate of magnesium hydroxyquinoline is filtered off, preferably on a sintered glass filter, and washed repeatedly with distilled water, then with 95 per cent alcohol. The precipitate is shaken with a liter or more of 50 per cent ethyl alcohol and kept in a dark bottle. A small portion is filtered off before being used.

Standard solution of magnesium. A stock solution of magnesium is prepared by dissolving 460 mg. of pure ribbon magnesium in a minimum of HCl and diluting to a liter. To 5 cc. of this stock solution are added 15 gm. of pure trichloroacetic acid and 2 gm. of ammonium oxalate and water to make up 500 cc. The resulting solution is equivalent to a trichloroacetic acid filtrate from serum containing 2.3 mg. of Mg per 100 cc. Magnesium is determined in this standard solution by the method described below. The average of a series of determinations is used for the standardization of the standard hydroxyquinoline solution.

Standard hydroxyquinoline solution. 0.18 gm. of purified 8-hydroxyquinoline is dissolved in a liter of water. Solution is made complete by thorough shaking and allowing to stand overnight. Filtration is unnecessary. The solution is then standardized by comparing the color obtained with 2 cc. samples with that from magnesium hydroxyquinoline precipitated from 6 cc. of the standard magnesium solution. This solution remains unchanged for at least a month, if kept in a dark bottle away from sunlight.

0.01 N HCl.

0.5 per cent $FeCl_3$ in 0.01 N HCl.

Special centrifuge tubes. The conical end of the ordinary 15 cc. centrifuge tube is drawn out to make a closed capillary tube about 5 mm. long and about 2 mm. in diameter. It is preferable to have a slight constriction at the junction of the capillary por-

tion with the body of the tube. The tube is marked for 6 cc. In such a centrifuge tube adequate drainage is possible without loss of precipitate because the latter is stoppered in the capillary tube by the drop of retained fluid. This tube was first described in the author's method for serum sulfate (13) and has been found advantageous for centrifugation of other minute precipitates that do not pack well.

Platinum-tipped stirring rods. A 1 cm. piece of platinum wire (about 20 gage) is sealed to the end of a 3 mm. glass rod. Such a stirring rod reaches into the capillary ends of the centrifuge tubes and allows thorough washing of the precipitate. Glass rods drawn out to a fine tip may also be used, but to employ them is hazardous for they tend to break off during stirring and may plug up the capillary end of the centrifuge tube.

Procedure

Calcium is precipitated from serum, as in the Kramer and Tisdall method, by addition of exactly 2 cc. of water and 2 cc. of serum to 1 cc. of 4 per cent ammonium oxalate in the special centrifuge tube. After an hour, the tube is centrifuged and as much as possible of the filtrate obtained. The material from duplicate determinations should furnish at least 9 cc. To this filtrate an equal volume of 8 per cent trichloroacetic acid is added with thorough stirring. The mixture is filtered or centrifuged. At least 12 cc. of the protein-free, calcium-free filtrate are obtained; this is now ready for duplicate magnesium determinations.

6 cc. of the filtrate, representing 1.2 cc. of serum, are transferred to the special centrifuge tube. 4 drops of the 6 per cent hydroxy-quinoline in alcohol are added and the mixture stirred with the platinum-tipped stirring rod. Then 10 drops of concentrated ammonia are added, which is 5 or 6 drops more than enough to make the solution alkaline. The mixture is thoroughly stirred and placed in a water bath at about 75° for 2 or 3 minutes, until clouding occurs. The stirring rod is then carefully washed off with 95 per cent alcohol from a wash bottle, so that the alcohol forms a layer about 1 cm. high. This layer prevents the rise of the precipitate along the sides of the tube. It is this tendency of magnesium hydroxyquinoline, like that of flowers of sulfur, to escape from watery solutions by rising above the surface of the

liquid that makes it very difficult to centrifuge or filter it quanti-
tatively. The precipitation is completed by replacing the tube in
the water bath at about 75° for 20 minutes, during which time
flocculation of the greenish yellow precipitate occurs. The tube
is centrifuged for 15 minutes at about 2000 R.P.M., inverted, and
drained for at least 2 minutes. The precipitate is washed with 10
cc. of the saturated solution of magnesium hydroxyquinoline in 50
per cent alcohol. The stirring rod is again washed off carefully
with 95 per cent alcohol to form a layer about 1 cm. deep. The
tube is again centrifuged for 10 minutes, inverted, and drained.
If the drainage is good, one washing is sufficient, but a second
washing usually secures more consistent determinations.

The washed precipitate is dissolved by adding exactly 1 cc. of
0.01 N HCl and stirring for 2 minutes in a boiling water bath.
Simultaneously, in another centrifuge tube, 2 cc. of standard
hydroxyquinoline solution are similarly treated for 2 minutes with
1 cc. of 0.01 N HCl. The solutions are allowed to cool to room
temperature and then treated with 1 cc. of the 0.5 per cent $FeCl_3$.
A dark green-blue color forms immediately. Water is added to
the 6 cc. mark. The solutions are thoroughly mixed and com-
pared in a colorimeter. The colors are easily matched, especially
in daylight. However, sunlight affects the color, at first intensify-
ing it, then changing the shade. Both standard and unknown
should therefore be exposed to minimal and equal amounts of sun-
light before being compared in the colorimeter. If at the time of
the determination no fresh standard solution of hydroxyquinoline
is available, a simultaneous determination of magnesium in the
standard magnesium solution can be run and compared with the
unknown.

The colors obtained by this method can be easily determined also
in a photoelectric colorimeter. Using the Cenco-Sheard-Sanford
photelometer with a blue filter, the author was able to obtain
a smooth curve, on semilogarithmic paper, which is practically
a straight line for the range of values fixed by the method (see
Fig. 1). The photoelectric colorimeter, of course, avoids the annoy-
ance of preparing fresh standard solutions. When the photoelec-
tric colorimeter is used, the final dilution should be made to 8 cc.
rather than to 6 cc. Readings of unknown solutions can be made
either directly from the curve or can be calculated from the equa-
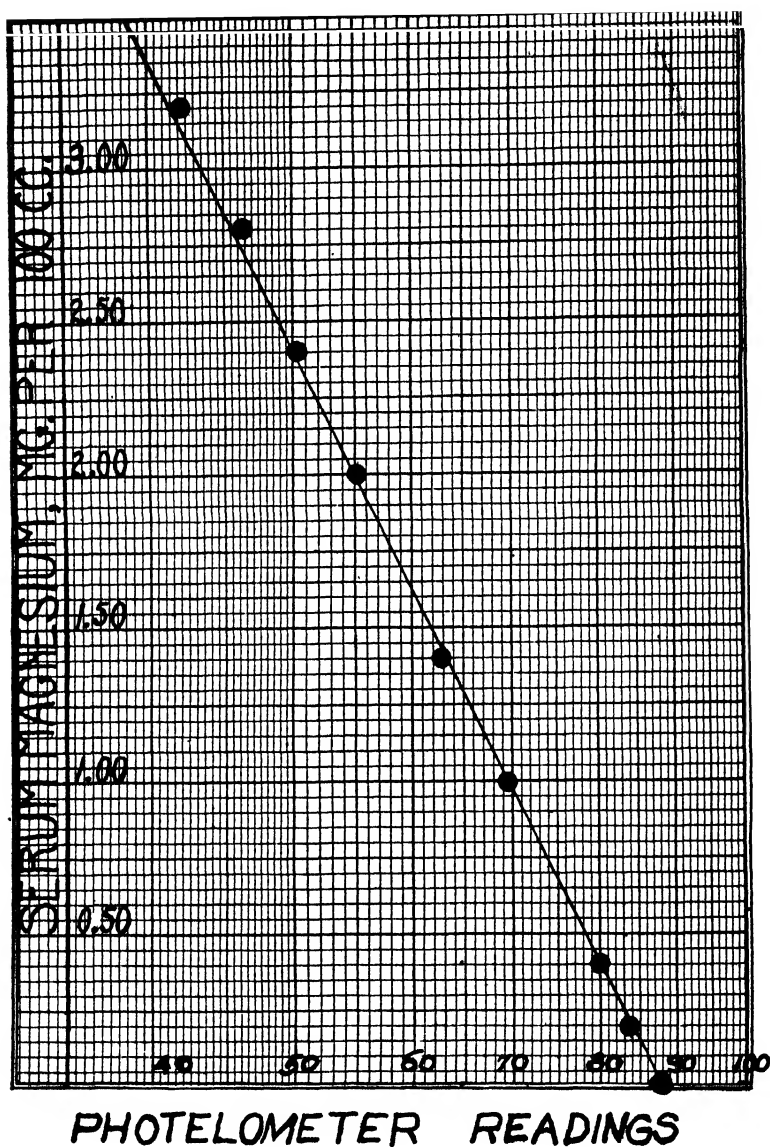
FIG. 1. Curve showing the relationship between photoelectric colorimeter readings and concentration of magnesium, as plotted on semilogarithmic paper.

tion of the curve which was found to be $C = 9.747 \log 88.0/R$ where C is the concentration of magnesium in mg. per 100 cc. of serum and R is the reading on the microammeter, when the reading for water is set at 100.

Comment on Method—Without the layering with alcohol, it was almost impossible to get consistently accurate results with the centrifugation technique. The alcohol must be added before flocculation has taken place, for after that it is too late to prevent the rise of portions of the precipitate along the walls of the tube above the surface of the liquid. On the other hand, the presence of alcohol seems to delay precipitation. Therefore, precipitation is allowed to take place first; then the alcohol is added.

In practically all previous hydroxyquinoline procedures washing is done with ammonia. This was avoided here, first because of the difficulty in preventing loss of precipitate and secondly because the pH for the final color formation must be controlled. The green-blue color diminishes in intensity in proportion to the hydrogen ion concentration and disappears altogether in strongly acid solutions. With the use of a neutral wash liquid and with the addition of a fixed amount of weak HCl, the final color is intense and proportional to the amount of hydroxyquinoline. It was found necessary to dissolve the magnesium hydroxyquinoline by heating with the HCl and then cooling, for if the ferric chloride solution is added directly to the precipitate a coating of iron hydroxyquinoline forms around particles of the precipitate and prevents completion of solution.

Nearly all users of the hydroxyquinoline method have employed NH_4Cl along with NH_4OH at the time of precipitation. In more concentrated solutions of magnesium, this procedure serves to prevent the precipitation of $Mg(OH)_2$. With filtrates from serum there is little danger of this occurring. At any rate, since a good concentration of ammonium ions is necessarily present as ammonium trichloroacetate, the further addition of NH_4Cl was deemed unnecessary. This conclusion was confirmed by controlled analyses with and without NH_4Cl. The danger of precipitation of $MgNH_4PO_4 \cdot 6H_2O$ along with magnesium hydroxyquinoline is remote, since the precipitation is carried out at 75° and is completed in several minutes.

In spite of the apparent complexity of the technique and the

number of precautions, the actual determination of magnesium by this method is very easy and rapid.   The accuracy of the

TABLE I

Accuracy of Determination of Magnesium

The results are expressed in mg. of Mg per 100 cc.

Sample	Magnes-ium	No. of determi-nations	Standard deviation	Added Mg	Total Mg	
					Calcu-lated	Found
			per cent			
Magnesium standard	2.30	32	±2.8			
Pooled serum	2.05	12	±3.6			
" "	2.20	16	±2.7			
" "	2.30	24	±4.2			
Serum	2.13			2.50	4.63	4.50
"	1.98			1.25	3.23	3.10
"	2.05			1.00	3.05	3.02
"	2.20			2.50	4.70	4.92
				5.00	7.20	7.28
"	2.26			2.50	4.76	4.64
				5.00	7.26	7.30

TABLE II

Concentration of Normal Serum Magnesium As Determined by Various Authors

Authors	Precipitating agent	Method	Range of values	Mean
			mg. per 100 cc.	*mg. per 100 cc.*
Watchhorn and McCance (14).................	Phosphate	Colorimetric	2.3 –2.66	
Becher (15)	"	"	1.8 –2.3	
Wacker and Fahrig (16)...	"	"	2.0 –2.6	2.28
Walker and Walker (17)....	"	"	1.6 –3.0	2.20
Bomskov (18)..	Hydroxy-quinoline	Bromination	1.7 –2.6	
Greenberg, Lucia, Mackey, and Tufts (19)...........	"	"	2.0 –3.66	2.74
Velluz and Velluz (20).....	"	"	1.60–2.40	2.00
Hoffman.................	"	Colorimetric	1.90–2.50	2.18

method is attested to by the fact that the standard deviation from the mean on multiple determinations was always below 5

per cent and frequently as low as 3 per cent (see Table I). Added magnesium was quantitatively recovered. Further proof of the reliability of the method is furnished by the fact that the determination of serum magnesium concentrations in thirty normal adults ranged between 1.90 and 2.50 mg. per 100 cc., with a mean of 2.18 mg. per 100 cc. and a standard deviation of ±0.15 mg. per 100 cc., which is within the range found by most previous workers (see Table II).

SUMMARY

A new colorimetric method for the determination of serum magnesium is described in which magnesium is precipitated as magnesium 8-hydroxyquinoline, and the hydroxyquinoline is estimated by measuring the green-blue color which it forms with ferric ions in weak HCl.

The applicability of photoelectric colorimetric determination of the iron hydroxyquinoline is demonstrated.

Serum magnesium concentration in thirty normal persons was found to be 2.18 ± 0.15 mg. per 100 cc.

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A MICROMETHOD FOR THE DETERMINATION OF STRONTIUM AND CALCIUM IN MIXTURES CONTAINING BOTH*

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A method for the estimation of small amounts of strontium and calcium in mixtures containing both is of increasing interest to the biochemist. Strontium has been used in studies of the mechanism of bone formation (1–6), and the knowledge thus gained has been applied to the correction of orthopedic deformities in children (7). The limitations of such investigations without a satisfactory analytical method cannot be developed here, but they are obvious to the workers using strontium salts.

The chemical procedures existing for strontium-calcium mixtures depend upon the compensation of errors (8, 9) and have not been applied to quantities of material of the order of 0.2 mg. There are, however, some spectroscopic methods which are sufficiently sensitive for quantities of such magnitude. These spectroscopic methods do not lend themselves to accurate quantitative measurements. They also require an expensive set-up and are technically difficult to use.

The most common method of separating strontium from calcium depends upon the relative insolubility of strontium nitrate in solvents such as absolute alcohol or concentrated nitric acid. The separation is not sharp, since strontium nitrate is somewhat soluble. This technique requires a number of delicate steps which make this principle unsuited to microquantities. A number of

other schemes have been proposed which have not survived the
test of experience. Among these is the method developed by
Sidersky (10) who precipitates calcium as the oxalate and stron-
tium as the sulfate with a mixture of ammonium sulfate and oxa-
late. This method has been criticized as unsatisfactory (11, 12)
because, under the conditions given, some of the strontium is
usually precipitated as the oxalate. Consequently, the separa-
tion is not quantitative. Separation by the differential solubilities
of the ferrocyanides (13) and dichromates (14) has also been re-
ported; but, these schemes are unsatisfactory. There is one proce-
dure described for small amounts in which the combined calcium
and strontium are precipitated as the oxalate, weighed, and subse-
quently titrated with standard permanganate (12). The relative
amount of each element is then calculated. This procedure
suffers from the defect that in removing adsorbed oxalate ions 2
per cent ammonia water is used. Since strontium oxalate is
soluble in this medium, results obtained are misleading unless a
correction is applied.

In the course of the present investigation an attempt was made
to separate calcium from strontium by experimenting with the
oxalates at various hydrogen ion concentrations. It was estab-
lished that quantities of calcium as small as 0.1 mg. may be
quantitatively precipitated as the oxalate at a pH as low as 3.0.
This was shown for macro quantities by Washburn and Shear (15).
Strontium, on the other hand, became increasingly more difficult
to precipitate quantitatively as the oxalate below pH 6.0. In
fact, no precipitate was observed at pH 3.0 for quantities of the
order of 0.1 to 0.2 mg. In the presence of calcium, however, some
of the strontium is precipitated, probably due to coprecipitation.
Consequently, this method of attack was not deemed suitable for
a quantitative separation. However, while the oxalate of stron-
tium was more difficult to precipitate, it was observed that the
sulfate of strontium is quite insoluble in a solution of dilute sul-
furic acid. Consequently, strontium and calcium mixtures were
treated with a mixture of sulfate and oxalate ions at pH 3.0. It
was established that, while calcium was quantitatively precipi-
tated as the oxalate, the strontium came down as the sulfate and
thus the calcium could be titrated quantitatively by means of
standard permanganate.

After developing the conditions for the estimation of calcium in the presence of strontium a means of determining both ions as the oxalate was sought. It was shown that the observation of Peters (16) holds for small amounts of strontium which may be quantitatively precipitated as the oxalate in a medium which is 20 per cent alcoholic at pH about 5.5. Calcium is of course readily precipitated as the oxalate under similar conditions, while magnesium is readily removed by a double precipitation. This precipitate cannot be determined oxidimetrically, since adsorbed oxalate ions are not removed by washing with an alcoholic solution, while a 2 per cent ammonia solution will dissolve some of the strontium oxalate. To avoid this difficulty the combined oxalates were converted to the carbonate by heat, which simultaneously removed by decomposition the adsorbed oxalate ions. The carbonates were treated with an excess of 0.01 N acid and titrated back with 0.01 N alkali. It was found necessary to avoid the use of standard sulfuric acid because strontium sulfate which is precipitated may adsorb some of the carbonate, thus causing errors. This solution could be used for the subsequent estimations of calcium by treating with a mixture of sulfate and oxalate ions at pH 3.0. For the sake of uniformity of technique the precipitated calcium was converted to the carbonate and determined acidimetrically.[1] Thus, one sample could be used for the determination of both strontium and calcium.

The principle of the method as finally developed is presented below.

(1) $Sr^{++} + Ca^{++} + C_2O_4^- \xrightarrow[\substack{\text{(by volume)}\\ \text{pH } 5.5}]{\text{20\% alcohol}} SrC_2O_4 + CaC_2O_4$

        $SrC_2O_4 + CaC_2O_4 \xrightarrow[\text{Heat}]{450\text{--}500°} \underset{\text{(SrO)}\quad\text{(CaO)}}{SrCO_3 + CaCO_3}$

(2) $Sr^{++} + Ca^{++} + C_2O_4^- + SO_4^- \xrightarrow[\text{pH } 3.0]{} SrSO_4 + CaC_2O_4$

        $SrSO_4 + CaC_2O_4 \xrightarrow[\text{Heat}]{450\text{--}500°} \underset{\text{(CaO)}}{SrSO_4 + CaCO_3}$

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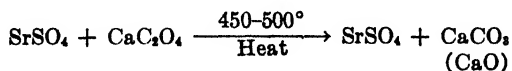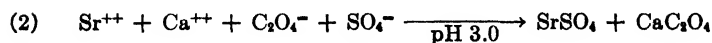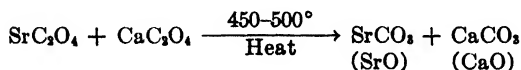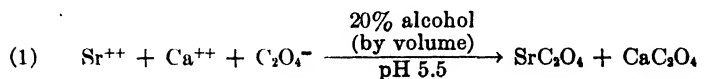
[1] Acidimetric titrations are used in some of the micromethods for estimating calcium (17–21).

At first, both the strontium and calcium ions are precipitated as the oxalates, which are then converted to the carbonates[2] at a temperature of 450–500° and the latter titrated acidimetrically.

Following this titration, the calcium ions are reprecipitated as the oxalate, while the strontium ions are precipitated as the sulfate. When the pH is slightly higher than 3.0, a double precipitation is necessary to remove the small amount of strontium that may come down as the oxalate. The calcium may be estimated oxidimetrically or acidimetrically.

The difference between the first and second titration represents the equivalents of strontium, while the second titration represents the equivalents of calcium.

It may be pointed out that any scheme that determines carbonate in small amounts may be used in this procedure.

Method

Reagents—All of the chemicals and solutions must be free from the presence of calcium and strontium. When these impurities are present, the reagent should be purified or a correction applied.

0.01 N hydrochloric acid. 100 cc. of a carefully prepared solution of 0.1 N acid are diluted with double distilled water to 1000 cc. This solution is kept in an automatic burette calibrated to 0.01 cc. The 0.01 N acid is stable if carefully handled.

. 0.01 N sodium hydroxide. 100 cc. of a carefully prepared solution of 0.1 N alkali are diluted with double distilled water to 1000 cc. This is kept in a paraffin-lined automatic burette calibrated to 0.01 cc., equipped with a soda-lime tube. The titer of the alkali must be carefully checked from time to time. The sodium hydroxide should be carbonate-free for best results.

Indicators. 0.04 per cent thymol blue, 0.04 per cent bromcresol purple, 0.04 per cent methyl red prepared according to Clark (22).

Ammonium oxalate reagent. A saturated solution of c.p. ammonium oxalate is prepared at 40–50° and allowed to cool to room temperature. The clear supernatant solution is used.

Oxalic and sulfuric acid mixture. The oxalic acid used is re-

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[2] If a higher temperature is used, oxides or a mixture of the carbonate and oxide may be formed. This, however, does not affect the titration because both are titrimetrically equivalent.

crystallized twice. The solution is prepared in such a manner that it is approximately molar with respect to sulfuric acid and 0.5 M with respect to oxalic acid. 73 gm. of oxalic acid and 100 gm. of concentrated c.p. sulfuric acid are made up to 1000 cc.

Dilute hydrochloric acid. An approximately 3 N hydrochloric acid is prepared by diluting the c.p. concentrated acid 3- to 4-fold.

0.25 N ammonium hydroxide. 2 cc. of c.p. concentrated ammonia water are diluted to 100 cc.

4 N ammonium hydroxide. 32 cc. of c.p. concentrated ammonia water are diluted to 100 cc.

95 per cent alcohol. Redistilled ethyl alcohol.

25 per cent alcohol. 100 cc. of 95 per cent alcohol are diluted to 400 cc.

Procedure

To 2.0 cc. of solution, in a 15 cc. Pyrex centrifuge tube, 1 cc. of ammonium oxalate and 1 drop of brom-cresol purple are added. This is washed down by 1 cc. of water and mixed. Following this, 1 cc. of 95 per cent alcohol is added drop by drop. The pH is adjusted to the gray color which is just between the yellow and purple of the indicator. The contents of the tube are allowed to stand overnight, after which it is centrifuged at about 2000 revolutions per minute for 10 minutes. The supernatant liquid is carefully aspirated off, with a drawn-out tube and rubber bulb, without disturbing the precipitate. The precipitate is suspended in 3 cc. of 25 per cent alcohol and centrifuged for 5 to 10 minutes. The washing and centrifuging are repeated twice. The washed precipitate is dried at 100–110° and heated over a Bunsen flame below red heat for 2 to 3 minutes or in an electric oven at 450–500° for 15 minutes. After this, an excess of 0.01 N hydrochloric acid is added (usually 3 cc.) and heated in a boiling water bath for 15 to 25 minutes. The excess acid is titrated back, while hot, in the presence of 1 drop of methyl red indicator. The difference between the acid and alkali used represents the combined titrimetric equivalent of strontium and calcium.

The above mixture contains all of the original calcium and strontium and may be therefore used for the determination of the titrimetric equivalent of calcium alone. When the time factor is important, an independent sample of 2 cc. may be used. The

solution is treated with 1 cc. of the oxalic-sulfuric acid solution and 3 drops of thymol blue. A sufficient amount of 4 N ammonium hydroxide is added until the pink of thymol blue is changed to that brown which is between the pink and yellow of this indicator. The tube is placed in a boiling water bath for 10 minutes and allowed to stand at room temperature for 90 minutes. It is then centrifuged and washed once with approximately 0.25 N ammonium hydroxide. The precipitate is then dissolved in a few drops of hot 3 N hydrochloric acid, followed by 1 cc. of the oxalic-sulfuric acid solution, the pH adjusted to 3.0, and then the mixture is allowed to stand as before and washed three times with 0.25 N ammonium hydroxide. The precipitate is heated and titrated acidimetrically as described in the previous paragraph. This represents the titrimetric equivalent of calcium alone.

Calculation of Results

Ca + Sr in equivalents = first titration
Ca " " = second "
Sr " " = first " minus second titration
1 cc. 0.01 N acid used = 0.2 mg. Ca
1 " 0.01 " " " = 0.44 " Sr

$$\text{Mg. per 100 cc.} = \frac{\text{cc. acid used} \times \text{equivalent weight}}{\text{cc. sample}}$$

Procedure in Presence of Magnesium—In the presence of small amounts of magnesium special precautions are not necessary. In the presence of relatively large amounts of magnesium the combined calcium and strontium oxalates are isolated by double precipitation. The precipitate, in a *graduated* centrifuge tube, is washed with 25 per cent alcohol, as described above, dissolved in a few drops of hot 3 N hydrochloric acid, neutralized to the first gray of brom-cresol purple with ammonium hydroxide, and made up to 2 cc. From this point on the technique described under the previous heading is used.

Procedure for Blood Serum—To 2 cc. of serum in a 15 cc. *graduated* Pyrex centrifuge tube 1 cc. of ammonium oxalate is added and the mixture is stirred. This is washed down by 1 cc. of water and mixed. Following this 1 cc. of 95 per cent alcohol is added drop by drop. (Important: protein will precipitate if the alcohol is not added dropwise with careful mixing.) It is then allowed to stand overnight, centrifuged, and washed with 25 per cent alcohol *three*

times. The precipitate is dissolved in a few drops of 3 N hydrochloric acid and then neutralized with ammonium hydroxide to the first gray of brom-cresol purple.   The volume is then made up to 2 cc. From this point the determination is carried out as described above for inorganic solutions.

<center>DISCUSSION</center>

Methyl red indicator, which is used in these titrations, is affected by carbonic acid.   To remove this possible source of error, the back titrations with the sodium hydroxide are carried out while hot.

The color of methyl red interferes with the adjustment of the pH to 3.0 by the use of thymol blue.   To overcome this difficulty a higher concentration of thymol blue is used, the pH is kept slightly above 3.0, and double precipitation is employed.

Double precipitation, when calcium only is being estimated, is not necessary if the pH is exactly adjusted to 3.0.   However, when the pH is too low the calcium will not precipitate completely as the oxalate, while a higher pH will cause some of the strontium to precipitate as the oxalate.   In the latter case, a second precipitation will eliminate this error, while in the first case once the precipitate is washed the results will be inaccurate.   To play safe it is more practical to use a slightly higher pH than 3.0 and employ double precipitation.   When a relatively large amount of strontium is present, it will not be possible to dissolve it in a few drops of hot hydrochloric acid.   This, however, will not affect the results, since all of the oxalates will go into solution quite readily and one may reprecipitate as usual.   In estimating the calcium alone it is not necessary to convert to the carbonate.   One may employ the oxidimetric technique of Kramer and Tisdall (23).

The addition of the alcohol drop by drop is especially important for protein solutions such as serum, since otherwise high concentrations of alcohol will localize and denature the protein, causing it to precipitate.   This precipitated protein will interfere with the titrations.   For similar reasons it is advisable not to allow the serum to stand longer than overnight.   Double precipitation in the first step of the procedure is mainly resorted to in order to remove the proteins.   It is not necessary for ashed serum.

The method developed above is general in nature and will be

equally effective for larger amounts, where one would preferably modify the details of the technique.

TABLE I

Effect of Alcohol Concentration upon Precipitation of Strontium As Oxalate

Sr present	Sr found		
	9 per cent alcohol	12 per cent alcohol	20 per cent alcohol
mg.	*mg.*	*mg.*	*mg.*
0.198	0.132	0.158	0.194
	0.136	0.158	0.194

TABLE II

Titration of Strontium-Calcium Mixtures

Sr present	Ca present	Theoretical titration with 0.01 N acid	Actual titration with 0.01 N acid
mg.	*mg.*	*cc.*	*cc.*
0.100	0.098	0.72	0.73
			0.75
			0.73
			0.75
0.200	0.194	1.42	1.43
			1.44
			1.40
0.100	0.400	2.23	2.25
			2.19
			2.21
0.440	0.320	2.60	2.57
			2.56
			2.58
1.100	0.170	3.35	3.31
			3.33
			3.34
			3.34

Results

The effect of alcohol concentration upon the precipitation of strontium as the oxalate is illustrated in Table I.

It is seen that in a 20 per cent alcoholic solution (by volume) strontium is quantitatively precipitated. The values for calcium, determined in a 20 per cent alcoholic medium, are also quantita-

tive: Ca present, 0.200 mg.; Ca found, 0.200, 0.198, 0.196, 0.206, 0.200 mg.

The titrimetric equivalents of various strontium-calcium mixtures precipitated in a 20 per cent alcoholic solution are given in Table II.

It is seen in Table II that the titrimetric equivalents of the two elements are equal to the sum of the titrimetric equivalents of strontium and calcium alone.   The error is within 1 drop (0.04 cc.)

TABLE III

Titration of Calcium in Strontium-Calcium Mixtures

Sr present	Ca present	Theoretical titration with 0.01 N acid	Actual titration with 0.01 N acid
mg.	*mg.*	*cc.*	*cc.*
0.100	0.098	0.49	0.44
			0.51
			0.47
			0.47
0.200	0.194	0.97	1.01
			0.95
			0.95
0.100	0.400	2.00	1.97
			2.00
			1.95
0.440	0.320	1.60	1.60
			1.57
			1.57
			1.56
1.100	0.170	0.85	0.88
			0.88
			0.85
			0.87

of the theoretically expected titration.   Thus, it may be concluded that the titrimetric equivalent of strontium and calcium combined may be obtained under the above conditions.

Estimation of calcium alone treated with the oxalic-sulfuric acid reagent at pH 3.0 are as follows: Ca present, 0.200 mg.; Ca found, 0.198, 0.192, 0.202, 0.200, 0.200, 0.194 mg.

It is seen that the accuracy of calcium estimation under these conditions compares favorably with that of the Kramer-Tisdall method (23).

The titrimetric equivalent of calcium alone in the various strontium-calcium mixtures shown in Table II are presented in Table III.

It may be readily observed in Table III that the actual titrations are within 1 drop of that expected theoretically.

TABLE IV

Estimation of Strontium and Calcium in Mixtures Containing Both
The values are expressed in mg.

Sr present	Sr found	Deviation	Ca present	Ca found	Deviation
0.100	0.119	+0.019	0.098	0.095	−0.003
0.200	0.200	0.000	0.194	0.194	0.000
0.100	0.110	+0.010	0.400	0.394	−0 006
0.440	0.435	−0.005	0.320	0.316	−0.004
1.100	1.082	+0.018	0.170	0.174	+0.004

TABLE V

Determination of Strontium and Calcium on 2 Cc. of Solutions
The values are expressed in mg. per 100 cc.

Nature of specimen	Sr present	Sr found	Ca present	Ca found
Inorganic solution containing 500 mg. Mg per 100 cc.	10.0	10.8	9.7	9.7
Sr added to serum	9.9	9.0	8.3	8.1
" " " "	9.9	9.7	9.1	
Serum of animals suffering from rickets due to strontium				
Experiment 122		13.0		4.8
		13.3		5.0
" 123-A		9.0		6.4
		10.8		5.3
" 123-B		12.3		5.0

The actual strontium and calcium values, calculated from Tables II and III, are presented in Table IV.

The values in Table IV are calculated from the average results of the three to four titrations shown in Tables II and III. The greatest deviation for calcium from the theoretical is 0.006 mg., while that for strontium is 0.019 mg. As a rule calcium is determined more accurately by this method than strontium. This is

partially due to the smaller equivalent weight of calcium and partially due to the fact that calcium is estimated directly, while strontium is calculated from the difference of two titrations.

Table V presents the results of the determinations (1) in the presence of large amounts of magnesium, (2) recoveries of known amounts of added strontium to swine serum, (3) on the serum of animals on a rachitic diet where the $CaCO_3$ was substituted by an equivalent amount of $SrCO_3$.

It is seen in Table V that in the presence of large amounts of magnesium (50 times the weight of calcium or strontium present) the average calcium value of duplicate determinations is theoretical, while the value for strontium is within the experimental error; that is, 0.02 mg. It may be concluded that the presence of magnesium will not interfere with the accuracy of the determination under our conditions. In the serum studies the original calcium content was established as the average of three calcium determinations by the Kramer-Tisdall method (23). To another portion of the serum a known amount of strontium was added and the combined titrimetric equivalent determined. This was within a drop of the theoretically expected titration. Subsequent to this, the calcium alone was titrated. The results were again within a drop of the theoretically expected titration. The values calculated in terms of mg. per 100 cc. are given in the second and third lines of Table V. From this evidence it is justifiable to say that the technique outlined for serum establishes the calcium and strontium content of that fluid. The serum of strontium-fed rats contains about twice as much strontium as calcium, as shown in Table V. However, in terms of molar concentration, the ratio of strontium to calcium is about 1:1. Moreover, it is interesting to note that the combined molar concentrations of strontium and calcium are equal to that of calcium alone in the serum of a rat kept on the same diet except that $CaCO_3$ is substituted for the $SrCO_3$ (4).

SUMMARY

1. A quantitative method is described for the estimation of small amounts of strontium and calcium in mixtures containing both.

2. This is accomplished by precipitating both strontium and

calcium as the oxalates in 20 per cent alcohol.   The oxalates are converted to the carbonates or oxides by heating.   The carbonates or oxides are then titrated acidimetrically.   The solution, following this titration, is treated with a mixture of oxalate and sulfate ions at pH 3.0.   Calcium is precipitated as the oxalate, while strontium is precipitated as the sulfate.   The calcium oxalate is then converted to the carbonate or oxide and titrated acidimetrically.   The calcium oxalate may also be titrated oxidimetrically. The difference between the first and the second titration represents the strontium in equivalents, while the second titration represents the calcium in equivalents.

3. In the presence of relatively large amounts of magnesium the strontium and calcium oxalates are isolated by double precipitation instead of a single precipitation.   Good results are obtained even when 10.0 mg. of magnesium are present in a solution containing only 0.2 mg. of strontium and 0.2 mg. of calcium.

4. This method may be applied directly to serum.   The strontium and calcium oxalates are isolated by double precipitation in order to remove the proteins.   The rest of the method is similar to the usual procedure.

5. The sera of strontium-fed rats contains about twice as much strontium as calcium.   The sum of the molar concentration of calcium and strontium together is that given by calcium alone, when the strontium of the diet is replaced by calcium.

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A SEROLOGICALLY INACTIVE POLYSACCHARIDE ELABORATED BY MUCOID STRAINS OF GROUP A HEMOLYTIC STREPTOCOCCUS*

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It has been clearly demonstrated that immunologically reactive polysaccharides of individually distinct chemical structure are responsible for the characteristic type specificity and immunological behavior of encapsulated pathogenic microorganisms such as pneumococcus (1). The pneumococci also contain proteins which are serologically related throughout the entire group, a somatic polysaccharide, C, which is serologically reactive and characteristic for the entire group (2, 3), and produce a serologically inactive polysaccharide apparently related to chitin (3).

On the other hand, the type-specific substance of the Group A, or human pathogenic, hemolytic streptococcus has been shown to be of protein nature (4). The hemolytic streptococcus is also characterized by other group-specific proteins (4–6) and by a group-specific, somatic polysaccharide, C, characteristic for all Group A streptococci, but different from corresponding substances isolated from animal pathogenic strains of streptococcus (4) and from the C substance of pneumococcus.

Nevertheless, it is known that Group A hemolytic streptococci can exist in the mucoid phase (7). In this phase the organisms are encapsulated, and, when grown under appropriate conditions, produce large, watery colonies. Culturally and morphologically the mucoid phase of Group A organisms corresponds closely with that phase of pneumococci in which type-specific polysaccharides

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are demonstrable. In pneumococci this phase is commonly referred to as the S (smooth) variety, but it has been shown (8) that it should more appropriately be designated as the M (mucoid) phase.

Since it seemed reasonable to suppose that Group A hemolytic streptococci might also produce soluble polysaccharides while in the mucoid phase, it was attempted to isolate substances of this nature. As no serological test was available comparable to the highly sensitive precipitin reaction of pneumococcus type-specific polysaccharides, orientating experiments were made by merely following the mild procedures recently introduced for the isolation of the pneumococcus polysaccharides (9). Since a definite result was obtained, the method was applied throughout the work.

<div align="center">EXPERIMENTAL</div>

Isolation of Polysaccharide from Broth Cultures—10 liters of peptone, meat infusion, phosphate broth containing 0.15 per cent of glucose were seeded with an 8 hour culture of hemolytic streptococcus of Group A Strain C-203 (Griffith Type 1 (10)) or Type 3 or 10 (N. Y. 5) in the mucoid phase. After 24 hours at 37° the culture was passed through a Sharples centrifuge and the effluent sterilized by the addition of 1 per cent of phenol. The liquid was concentrated *in vacuo* to 0.1 volume, the temperature being kept below 35°. 100 gm. of crystalline sodium acetate and 25 ml. of glacial acetic acid were added and the polysaccharide was precipitated with 1.25 volumes of alcohol. After the precipitate had settled the supernatant liquid was poured off and discarded. The precipitate settled compactly on centrifugation, and in some cases a syrupy third layer formed and was discarded. The solid was dissolved in 200 ml. of water and the solution was tested with iodine. When glycogen was present, it was removed at pH 6.5 with a little saliva. 10 gm. of sodium acetate and 5 ml. of glacial acetic acid were added and the solution was shaken mechanically with 50 ml. of chloroform and 5 ml. of butyl alcohol (9, 11). Shaking with fresh portions of solvents was repeated as long as a solid emulsion layer remained after centrifugation. The polysaccharide was then precipitated by the addition of 1.25 volumes of alcohol. The precipitate was dissolved in 100 ml. of 5 per cent sodium acetate containing 2.5 ml. of glacial acetic acid, and re-

precipitated as before until the material was phosphate-free.   The
polysaccharide was then dissolved in 100 ml. of water and 500 ml.
of redistilled alcohol were added.   If necessary a small amount of
sodium acetate solution was added to induce flocculation.   The
precipitate was washed several times with redistilled alcohol,
filtered, and dried.   The yield of polysaccharide isolated in this
way as the neutral sodium salt was from 60 to 100 mg. per liter of
culture for Type 1 hemolytic streptococcus, 60 to 140 mg. per liter
for Type 3, and 50 mg. per liter for Type 10.

Isolation of Polysaccharide from Growth on Agar—Since the mu-
coid phase of hemolytic streptococci is often better maintained on
suitable solid media, Strain C-203 was grown on neopeptone-blood-
agar plates at 37° for 12 hours.   The saline washings were mixed
with an equal volume of alcohol in order to kill the streptococci,
let stand for several days in the cold, and worked up much as in
the previous instances, except that care was taken to remove
numerous agar fragments, and the polysaccharide was precipi-
tated twice from aqueous solution with 5 volumes of glacial acetic
acid.   The yield was small, so that a complete comparison could
not be made with the polysaccharide obtained from the broth cul-
tures.   The products appeared, however, to be similar (Table I).

*Recovery of Polysaccharide from Hemolytic Streptococci in R
Phase (8)*—A 24 hour broth culture of Strain C-203 in the R phase
was worked up as described above.   Throughout this preparation it
was necessary to use 1.75 volumes of alcohol to precipitate the
polysaccharide instead of 1.25 volumes.   The yield was only 9 mg.
per liter of material similar to that isolated from the mucoid or M
cultures (Table I).

Attempt to Isolate Polysaccharide from Sterile Broth—Since no
direct method was available for identifying the products obtained as
constituents of the hemolytic streptococcus, it was necessary to
find out whether or not similar substances occurred in the culture
medium.   3 liters of the same lot of broth used for Type 3
streptococcus were concentrated to 300 ml. *in vacuo* and then
worked up in the same way.   1.5 volumes of alcohol were re-
quired to give a precipitate in the concentrated broth.   After
the removal of glycogen with saliva, protein by the chloroform
treatment, and salts by precipitation with alcohol and acetic acid
in the presence of sodium acetate, less than 10 mg. of a white

TABLE I

Properties of Mucoid Polysaccharide, Group A Hemolytic Streptococcus

	Ash as Na	Nitrogen*	$[\alpha]_D^*$	Neutral equivalent*	Acetyl*	Uronic anhydride*	Reducing sugar after hydrolysis*	ηrel. in H₂O†	ηrel. in 0.9 per cent NaCl‡
	per cent	*per cent*	*degrees*		*per cent*	*per cent*	*per cent*		
Griffith's									
Type 1, Strain C-203..........	5.3	3.5	−73	411§	11.0	41.1	75		
Same grown on agar..........		2.2	−53			+	65	25	
Type 1, Strain C-203 R.......		3.7	−16			+	52		
" 3..................	5.9	3.8	−77	367§	10.9	42.4	84	57	3.3
" 3, free acid........	0.0	3.1	−47	416		40			
" 10, Strain N. Y. 5....	5.8	3.8	−86	374§		42.6	82	15	2.0
Calculated for polysaccharide..	5.7	3.7		379	11.3	46.4			

Properties of polysaccharide isolated from mammalian sources (16)

	Ash as Na	Nitrogen*	$[\alpha]_D^*$	Neutral equivalent*	Acetyl*	Uronic anhydride*	Reducing sugar after hydrolysis*	ηrel. in H₂O†	ηrel. in 0.9 per cent NaCl‡
Bovine vitreous..............		3.1	−51	424	11.7	41.1	69.5		
Human umbilical cord.........		3.4	−65	391	13.4	44.1	62		

* Calculated to the ash-free basis, except for the agar-grown and R strain samples.
† Of 0.2 per cent solution in H₂O.
‡ Of 0.1 per cent solution in 0.9 per cent NaCl.
§ Calculated from the ash content.

solid was isolated. This material differed from the polysaccharides previously obtained in still giving a biuret reaction. It was not further examined.

Since it was still possible that a polysaccharide in the sterile broth had been lost in the process of purification, attempts were made to isolate it directly from the peptone and meat infusion used. 50 gm. of Bacto-Peptone were dissolved in 200 ml. of 10 per cent sodium acetate containing 5 ml. of glacial acetic acid. No precipitate formed on addition of 2 volumes of alcohol, while 3 volumes gave a slight turbidity that did not settle.

5 pounds of lean, ground beef were covered with distilled water, allowed to stand overnight in the ice box, heated to boiling, and filtered. After concentration to 350 ml. *in vacuo* 30 gm. of sodium acetate and 15 ml. of glacial acetic acid were added and the solution was treated with 500 ml. of alcohol. The heavy precipitate which formed was centrifuged off, dissolved in 150 ml. of water, and shaken for 1 hour with 25 ml. of chloroform and 5 ml. of butyl alcohol. After centrifugation the clear supernatant liquid was poured off and precipitated with 225 ml. of alcohol after addition of 10 gm. of sodium acetate. After the removal of phosphates and purification of the small amount of residual substance as in previous instances, the final solution became turbid on addition of 3 volumes of alcohol but yielded no precipitate.

Chemical and Physical Properties of Mucoid Streptococcus Polysaccharide—The properties of the preparations are summarized in Table I. Nitrogen was determined by the micro-Kjeldahl method, the acetyl content as described previously (9), uronic anhydride by the method of Burkhart, Baur, and Link (12), and reducing sugars after acid hydrolysis by the Hagedorn-Jensen method (13). Viscosities were determined in an Ostwald viscometer at 20°. In the calculation of the basic ash it was assumed that the only cation present was sodium. The neutral equivalent was calculated from the ash content, but as a check the free acid was prepared from 0.5 gm. of the sodium salt of the Type 3 polysaccharide. The chilled aqueous solution was acidified to Congo red with dilute HCl and precipitated with 1.5 volumes of redistilled alcohol. The precipitate was redissolved in water and reprecipitated twice with redistilled alcohol in the presence of a drop of 10 per cent HCl. The precipitate was finally filtered,

washed free from chloride with redistilled alcohol, and dried *in vacuo* over calcium chloride and sodium hydroxide pellets. The free acid was much more hygroscopic than the sodium salt and required prolonged heating at 60° *in vacuo* over P_2O_5 to reach constant weight. 20 mg. gave no weighable ash. The neutral equivalent, determined by titration, agreed with the values calculated from the sodium salt (Table I).

The polysaccharide samples contained no phosphorus, sulfur, or free amino nitrogen.

The preparation from Type 1 streptococcus was hydrolyzed with N H_2SO_4, $[\alpha]_D$ changing from an initial value of $-73°$ to $+19°$ at the end of 3.5 hours. The reducing power increased to 76 per cent. An attempt was made to isolate the uronic acid component as the barium salt, but it could not be separated from the nitrogen-containing portion by precipitation with alcohol.

250 mg. of the ash-free Type 3 polysaccharide acid were refluxed for 6 hours with 18 per cent HCl in the apparatus used for the determination of uronic acid. The CO_2 evolved was equivalent to 99.6 mg. of uronic acid. The hydrolysate was decolorized with norit and concentrated *in vacuo* to a volume of 5 ml. After it had stood overnight in the ice box the crystalline deposit was filtered off. A second crop was obtained by addition of acetone to the mother liquor. The two portions were combined and recrystallized from 80 per cent methyl alcohol. Yield, 88.5 mg., or 75 per cent of the amount calculated from the N content. Calculated for $C_6H_{13}O_5N \cdot HCl$, N 6.5, Cl 16.5 per cent; found, N (Dumas) 6.2, Cl 16.5 per cent.[1] $[\alpha]_D$ (initial), $+98°$; final, $+68°$.

The substance was therefore glucosamine hydrochloride.

0.25 gm. of the sodium salt of the Type 3 substance, oxidized with nitric acid according to van der Haar (14), yielded an impure specimen of what appeared to be potassium acid saccharate, indicating the presence of glucuronic acid. Mucic acid was not found.

Sera from rabbits and horses immunized with hemolytic streptococcus in the mucoid phase failed to give a precipitin reaction with the mucoid polysaccharide isolated from either homologous or heterologous strains.

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[1] These analyses were made by Mr. William Saschek of the Department of Biological Chemistry.

Rabbit antisera against Types 1 (Strain C-203), 14, and 23 mucoid hemolytic streptococci failed also to fix complement with the mucoid polysaccharides of Types 1, 3, and 10 (experiments by Dr. A. J. Weil).

DISCUSSION

From the experiments recorded above it is evident that the mucoid phase of Group A hemolytic streptococcus elaborates a soluble polysaccharide when grown in broth culture. A similar carbohydrate was also isolated from agar growths of the organisms, but in too small an amount to permit rigorous purification. Nevertheless, it resembled the polysaccharide from the broth culture in its optical rotation, nitrogen content, positive test for uronic acid, and the high viscosity of its solutions. With the aid of a higher proportion of alcohol as precipitant a small amount of carbohydrate was also isolated from a broth culture of hemolytic streptococci in the R phase. This resembled the mucoid material in nitrogen and uronic acid content, but differed in its lower optical rotation, far lower viscosity, and in the greatly reduced yield. The broth culture medium and its components, Bacto-Peptone and lean beef, failed to yield appreciable quantities of polysaccharide when worked up even more rigorously than the streptococcus material.

. The properties of the various lots of polysaccharide obtained from three different types of Group A hemolytic streptococci in the mucoid phase correspond in general within the limit of error of the analytical methods used and according to the rather limited criteria of purity which can be applied to products of this kind. The conclusion therefore seems justified that Types 1, 3, and 10 of Group A hemolytic streptococci produce the same polysaccharide. The greatest difference appears in the higher viscosity of the Type 3 preparations. This may, however, be explained by an increased degree of polymerization in view of the identity of the other properties.

The analytical data agree well with those calculated for a polysaccharide composed of equal numbers of N-acetylglucosamine and glucuronic acid units, as shown in Table I. The substance thus differs from the serologically inactive polyglucose obtained by Oerskov (15) from the mucoid material accompanying growth

of a non-hemolytic strain of streptococcus isolated from mice. The hemolytic streptococcus polysaccharide, however, appears identical with that isolated by Meyer and Palmer (16) from bovine vitreous humor and the Wharton's jelly of human umbilical cord, as will be seen by comparison with data from their publication given in the last two lines of Table I. By no test has it been possible to distinguish between the carbohydrate isolated from streptococcus and that from mammalian sources, and additional evidence for their identity is presented in the accompanying paper by Meyer, Dubos, and Smyth (17). While glycogen has long been known to occur in the complex mixture of polysaccharides elaborated by tubercle bacilli (18) and possibly by pneumococcus (19), it is believed that this is the only other known instance of the formation of a polysaccharide both by a microorganism and by the animal body. The failure of the polysaccharide to function as an antigen or hapten and stimulate the formation of antibodies might thus be accounted for, since it is a substance which occurs normally in the mammalian organism.

A comparison with other bacterial polysaccharides shows that the mucoid streptococcus carbohydrate resembles the specific polysaccharides of pneumococcus in being associated with the encapsulated phase of the microorganism and is like the Types I and III·pneumococcus substances in its high content of uronic acid. It differs from these polysaccharides, however, in its failure to react with the sera of animals injected with the streptococci and in its lack of type specificity, the same substance having been isolated from three different types of Group A hemolytic streptococci. It will be recalled that the pneumococcus capsular polysaccharides are chemically, as well as serologically, different for each type. The new carbohydrate differs markedly from the serologically reactive, somatic C polysaccharide of Group A hemolytic streptococci, since it contains glucuronic acid and is phosphorus-free, while the C substance gives no test for uronic acid and contains phosphorus.[2]

The study of the polysaccharide of the mucoid hemolytic streptococcus is being continued.

[2] Unpublished results.

SUMMARY

1. A method is given for the isolation of a serologically inactive polysaccharide from cultures of three types of Group A hemolytic streptococci in the mucoid phase.   The substance was not found in the media used.

2. The polysaccharide, composed of N-acetylglucosamine and glucuronic acid units, appears to be identical with that occurring in bovine vitreous humor and human umbilical cord.

3. The significance of these findings is discussed.

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THE HYDROLYSIS OF THE POLYSACCHARIDE ACIDS OF VITREOUS HUMOR, OF UMBILICAL CORD, AND OF STREPTOCOCCUS BY THE AUTOLYTIC ENZYME OF PNEUMOCOCCUS*

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It has been shown by Avery and Cullen that the lysis of pneumococci is due to an autolytic enzyme present in all types of pneumococci (1). Moreover this enzyme acts upon heat-killed virulent pneumococci in at least two ways: (1) it renders the cells Gram-negative, and (2) it renders them unable to bring about the production—in the serum of rabbits immunized by the intravenous route—of the type-specific precipitin directed against the capsular polysaccharide of the strain used (2). However the hapten properties of type-specific carbohydrates are not affected by the enzyme, nor are these polysaccharides chemically altered.

In a study of the enzymatic properties of this autolysin it was found that it hydrolyzed the polysaccharide acids from vitreous humor (hyaluronic acid), umbilical cord, and streptococcus. The tissue polysaccharides were similar in composition, rotation, and general physical properties. Glucuronic acid, glucosamine, and acetyl were present in about equimolar concentration (3). The third polysaccharide acid, isolated by Kendall, Heidelberger, and Dawson (4) from strains of Group A streptococci, resembled the other two acids in composition and rotation. Their hydrolysis by this autolysin and the kinetics of the reaction of all three further emphasize their identity.

The action of the bacterial enzyme is quite specific, since a

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* A part of this work appeared as a preliminary note (*Proc. Soc. Exp. Biol. and Med.*, **34**, 816 (1936)).

polysaccharide from gastric mucin, containing acetylglucosamine and galactose ((3) foot-note 7), and chondroitinsulfuric acid were not affected. Nor had commercial trypsin, saliva, takadiastase, lysozyme, and emulsin any action on these three polysaccharide acids.

An enzyme similar in action was found in the tissues of ciliary body and iris. The tissue enzyme here may have the important biological function of constantly removing from the aqueous humor any excess of hyaluronic acid normally present in small quantities (5).

In this paper evidence will be presented which suggests that the same enzyme or enzyme complex of the autolytic system of pneumococcus is responsible for the hydrolysis of the polysaccharide acids as for the lytic action on the heat-killed pneumococci.

EXPERIMENTAL

The polysaccharide acids from vitreous humor and umbilical cord were prepared according to previously published methods (3). It made no difference whether viscous or non-viscous solutions of the sugars were used for the experiments. Two different samples of polysaccharide acids from streptococcus were obtained from Dr. Heidelberger's laboratory. The enzyme used was obtained from autolysates of pneumococci (2).

In the first experiment an example is given of the hydrolysis of the three polysaccharides by dilutions of the same enzyme preparation (Fig. 1).

It is evident from this experiment that the kinetics of the reaction are about the same for the three substrates, the slight differences being due undoubtedly to difference in purity of the preparations.

The influence of the pH was determined on 10 mg. samples of the polysaccharides in 1 cc. of saline solution incubated with 1 mg. of pneumococcus enzyme in different buffer solutions varying from pH 4 to 8. The pH of the final solutions was measured with the glass electrode. The optimal rate of hydrolysis was observed at about pH 5.8 with citrate or phosphate buffer, and the reaction did not take place below pH 4.5 nor above pH 8. Likewise the action of the enzyme on heat-killed pneumococci could be obtained between pH 5 and 8 with an optimum at pH ∼ 6.
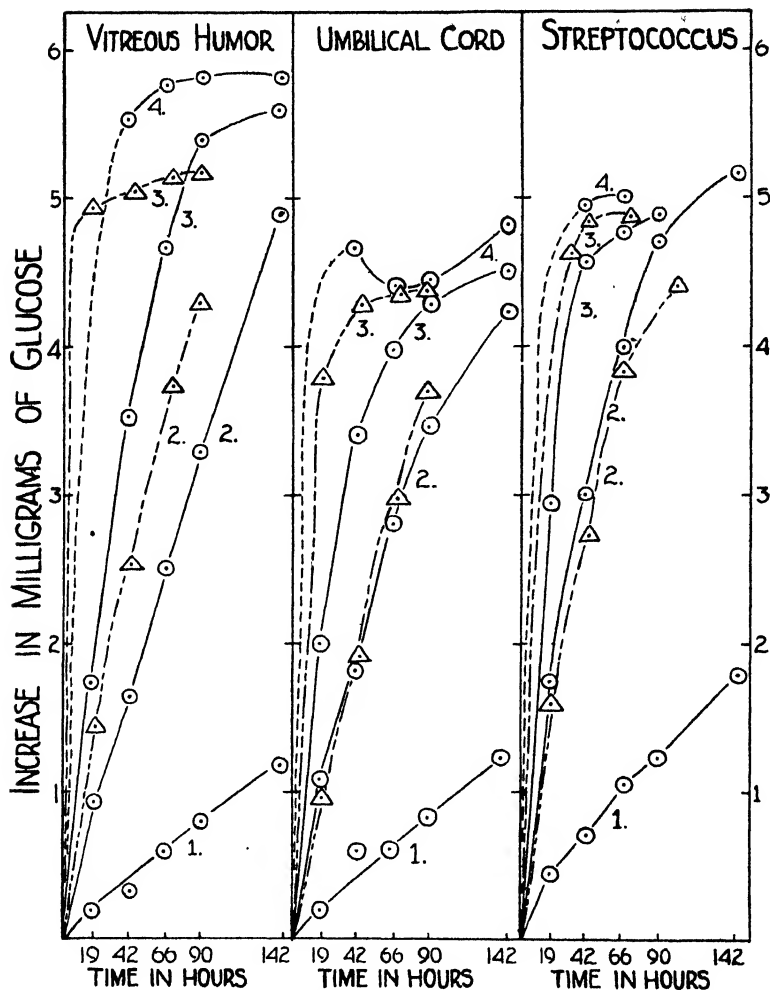
FIG. 1. Comparison of three polysaccharide acids incubated with varying concentrations of enzyme. The increase of reducing sugar (Hagedorn-Jensen) was determined on 10 mg. samples of polysaccharide acids in 1 cc. of saline incubated with varying amounts of pneumococcus enzyme in 1 cc. of citrate buffer, pH 6.0, at 37°. Toluene was added to all samples to prevent bacterial growth. The solid lines represent the first experiment; broken lines, second experiment. The numbers denote the concentration of the enzyme solution: *1*, 0.05 per cent; *2*, 0.10 per cent; *3*, 0.50 per cent; *4*, 1.00 per cent. All values are corrected for the reducing substances in the incubated enzyme and substrate controls.
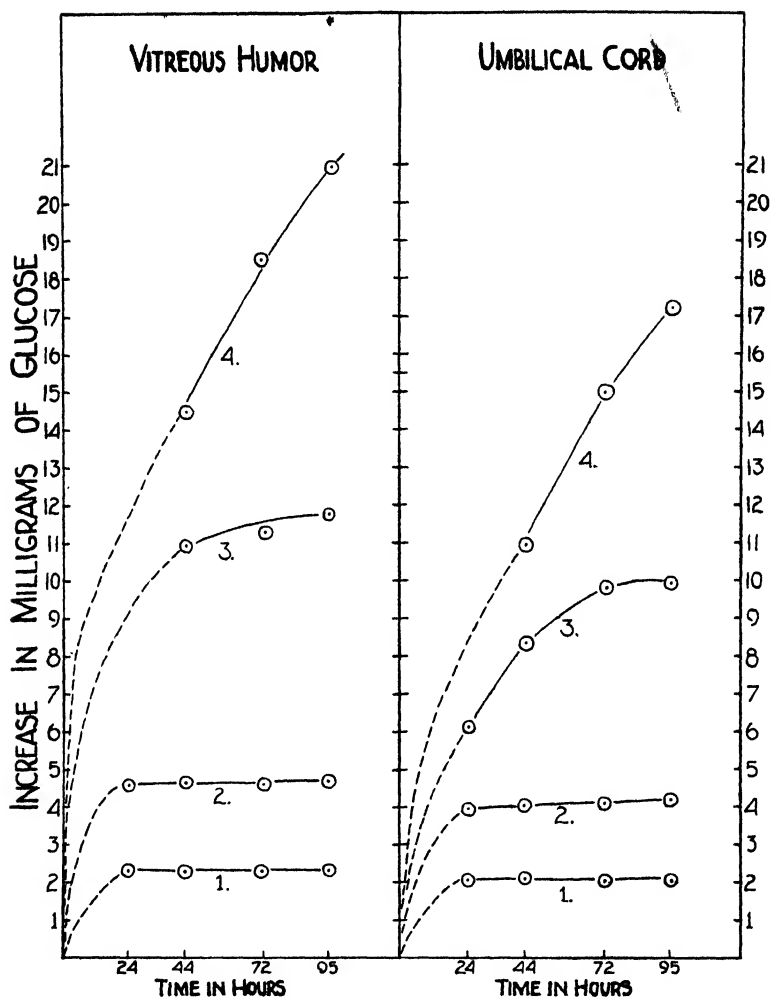
Fig. 2. Influence of substrate concentration. The increase of reducing sugar (Hagedorn-Jensen) was determined on varying amounts of polysaccharide acids in 1 cc. of saline incubated with 5 mg. of pneumococcus enzyme in 1 cc. of citrate buffer, pH 6.0, at 37°. Toluene was added to all samples to prevent bacterial growth. The numbers denote the concentration of the substrate solution: *1*, 0.5 per cent; *2*, 1.0 per cent; *3*, 2.5 per cent; *4*, 5.0 per cent. All values are corrected for the reducing substances in the incubated enzyme and substrate controls.

Fig. 2 shows the influence of the substrate concentration. The percentage of the sugar hydrolyzed is about the same for all concentrations, the only difference being that the maximum is reached more slowly with higher concentrations of substrate. Addition of acetylglucosamine, glucosamine, or glucuronic acid had no influence on the kinetics of the reaction. The end-point with purer preparations reaches about 70 per cent of the theoretical reducing value.

Inactivation by iodine and partial reactivation by sulfite and arsenite are shown in Table I. In this experiment reduction was estimated by the Folin-Wu copper method. The iodometric titration of the Hagedorn-Jensen method proved unsatisfactory in the presence of the reagents used, since variable blank values

TABLE I

Inactivation by Iodine and Partial Reactivation by Sulfite and Arsenite

Mixture No.	Mixture	Increase of glucose after 42 hrs. incubation at 37°
		mg.
1	10 mg. vitreous polysaccharide + 5 mg. enzyme	4.92
2	Mixture 1 + 0.125 cc. 0.1 N I$_2$ in 2.5% KI	0.25
3	" 2 + (after 10 min.) 1 cc. 0.1 N Na$_2$SO$_3$ (neutralized)	1.06
4	Mixture 2 + (after 10 min.) 1 cc. 0.1 N As$_2$O$_3$	0.68

were obtained after removal of the excess of iodine or sulfite; whereas in the Folin-Wu method no reduction was measurable in the blanks.

Iodine likewise inhibits the action of the enzyme on the pneumococci. This inactivation is completely reversible under certain conditions by reducing agents such as thioglycolic acid.

Incubation at 60° for 10 minutes decreases the activity of the enzyme by 84 per cent. Heating the enzyme solution just to boiling abolishes its action entirely. Under the same conditions the enzyme also loses its activity against heat-killed pneumococci.

In order to test whether the polysaccharide acids had any inhibiting influence upon the action of the enzyme on the heat-killed pneumococci, the following experiment was performed. The enzyme was incubated with the polysaccharide acids of

vitreous humor and umbilical cord (the polysaccharide of streptococcus was not tried) in the following proportions.

Solution	Polysaccharide acid in 2 cc. of buffer, pH 6.0, added to 0.1 cc. of enzyme
A	None
B	5 mg. vitreous polysaccharide
C	2 " " "
D	5 " umbilical cord polysaccharide

After 7 hours incubation at 37° the different solutions were added, as described in Table II, to 1 mg. of heat-killed pneumococci suspended in 2 cc. of buffer solution at pH 7.0.   Table II shows the approximate number of cocci which had become Gram-negative after 18 hours incubation at 37°.   Table II shows that the enzyme

TABLE II

Cocci Made Gram-Negative by Various Enzyme Solutions Incubated for 18 Hours at 37°

Solution	Gram-negative
cc.	
0.02 A	All
0.01 "	About half
0.02 B	None
0.01 "	"
0.02 C	About half
0.04 "	All
0.02 D	None
H_2O	"

solution incubated with buffer alone was of such concentration that it just sufficed to make all organisms Gram-negative.   The higher concentration of vitreous humor and umbilical cord entirely prevented the action of the enzyme upon the pneumococci; a lower concentration prevented it not at all or only partially. When, however, a mixture of enzyme and heat-killed pneumococci was added to the polysaccharide acids, no inhibition could be observed.

DISCUSSION

The evidence here presented shows that the hydrolysis of the polysaccharide acids is due to the same enzyme of the autolytic system as that which is responsible for the lysis of the heat-killed

pneumococci. The factors pointing to this conclusion are the similar pH curves, temperature inactivation point, and reversible oxidation and reduction, also, most important, the inhibition of the enzyme action on the bacteria by the polysaccharides. One might explain this specific inhibition by a fixation of the enzyme by the excess of isolated polysaccharide acids sufficient to prevent its action on a hypothetical substrate in the bacterial membrane. Such a substrate has not yet been found. It should be present in all types of pneumococci (since the enzyme is not type-specific). This sugar might be lost by hydrolysis during the autolysis, since autolyzed cultures are usually used for the isolation of the polysaccharides of pneumococcus. Two polysaccharides of pneumococci, obtained by Dr. Heidelberger, proved to be completely resistant to the enzyme. The one was a non-antigenic polysaccharide, obtained from Type IV pneumococcus (6) the other was a preparation of the C substance (6, 7). The latter was of special interest, since it is a substance known to be present in all types of pneumococcus. An attempt will be made to obtain a non-type-specific substrate from killed pneumococci, which should be hydrolyzed by the lysin.

In previous work (8) it was suggested that the action of lysozyme, a lytic enzyme present in egg white, tears, and susceptible *Sarcinæ*, was due likewise to a hydrolysis of a sugar present in the membrane of the bacteria. A similar sugar must be present in an egg mucoid fraction, since preparations of egg mucoid were also hydrolyzed by lysozyme. Lysozyme, like the pneumococcus lysin, was reversibly oxidized by iodine.

As with lysozyme, flavianic acid could be used advantageously to precipitate the pneumococcus enzyme from acidified and filtered solution. The precipitate was redissolved in alkali and reprecipitated by acid. Some foreign protein, salts, and carbohydrates thus could be removed. This flavianic acid-enzyme complex was highly active both against the bacteria and the polysaccharides. In contrast to lysozyme, the enzyme was inactivated by alcohol and acetone.

SUMMARY

The lytic enzyme of pneumococcus hydrolyzes three polysaccharide acids of apparently identical structure, obtained from

vitreous humor, umbilical cord, and Group A streptococcus. The action of the same lysin on pneumococci seems to be due to a hydrolysis of a similar substrate present in the pneumococcus cell. Such a substrate is at present unknown.

The similarity of the action on the three isolated polysaccharides and on the pneumococcus is shown by (1) their pH optimum, (2) their heat inactivation at the same temperature, (3) their reversible oxidation by iodine, and (4) by the inhibition of the action of the pneumococcus vaccine by the polysaccharide acids of vitreous humor and umbilical cord.

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SPECIFIC AND NON-SPECIFIC CELL POLYSACCHARIDES OF A HUMAN STRAIN OF TUBERCLE BACILLUS, H-37

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In recent years immunologically reactive material of carbohydrate nature has been isolated from human type tubercle bacillus cells or culture filtrate by a number of workers (1–11). The present writers have briefly reported the isolation of two immunologically distinct polysaccharides from cells of a human strain, H-37, of the tubercle bacillus (7, 11). Since such far reaching fractionation has not been reported by others, it would appear justifiable to outline in greater detail some of the experiments made, the methods of fractionation used, and the products obtained from the complex carbohydrate mixture in the initial extract.

EXPERIMENTAL

Separation of Polysaccharide Fractions from Strain H-37 Tubercle Bacillus Cells

Of the various procedures adopted and modified since the inception of the work, only the one now in use will be given, since it dispenses with alkali, mineral acid, and the use of heat.

600 gm. of dried, defatted, pulverized bacilli were extracted with chloroform in order to remove much wax, filtered in a large Buchner funnel, and washed several times with alcohol. While still moist, the mass was transferred to a percolator provided at the bottom with a filter plate covered with asbestos and a layer of purified sand, and was further dried by suction. Percolation was started with 1 liter of 3.5 per cent acetic acid and this was followed

by 0.35 per cent acetic acid containing 0.5 per cent of phenol.   In case percolation was too rapid, a stopper was inserted in the bottom of the percolator for several days.   Occasionally percolation was exceedingly slow and it was necessary to siphon off the acid extract repeatedly and centrifuge.

When the neutralized extract gave only a weak reaction after 24 hours with antibacillary serum, usually after 25 to 35 liters of percolate had been collected, the solution was concentrated *in vacuo* to about 2 liters and centrifuged.   The supernatant liquid was again concentrated *in vacuo* to about 200 cc. and precipitated by pouring into about 2 liters of alcohol.   The precipitate was dissolved in water, centrifuged, and the residue washed repeatedly with 10 to 20 per cent alcohol containing sodium acetate and made just acid with acetic acid.   Before addition to the brownish supernatant liquid the turbid washings were combined, concentrated *in vacuo*, shaken with chloroform (*cf.* (12)), centrifuged, and run through V and W Berkefeld filters which had been washed with alkali and finally with acidified sodium acetate solution.   The filtrate was concentrated *in vacuo* and combined with the main supernatant liquid, as was also a fraction obtained from the original alcoholic supernatant by concentration to a syrup, reprecipitation with alcohol, and solution in water.   The glycogen present (*cf.* (1)) was removed by addition of a little saliva, and, after the test with iodine was negative, the combined solutions, at a volume of 60 cc., were treated with acetic acid until no more precipitation occurred, 1.25 liters being required.   The precipitate was centrifuged off, washed with acetone, and dried *in vacuo*.[1]   Precipitation with glacial acetic acid was repeated twice, the volumes of water and acetic acid used being 50 cc. and 1.25 liters for the first reprecipitation and 35 cc. and 750 cc. for the second.   Except in the last instance the supernatant liquids from the acetic acid precipitates (Fraction I) yielded much solid material on addition of acetone (Fraction II).

The acetic acid-insoluble portion (Fraction I) was dissolved in 30 cc. of water, and 90 cc. of methyl alcohol were added.   The fraction (C)[2] thus precipitated in 75 per cent methyl alcohol was

[1] All precipitates were washed with acetone and dried *in vacuo* before being redissolved, unless otherwise stated.

[2] The lettering of the fractions is in accordance with earlier usage in this laboratory, rather than in the order of separation.

centrifuged off and the methyl alcohol concentration brought up to 90 per cent. The precipitate (B_2) was centrifuged off and an additional fraction (B_1) obtained by pouring the supernatant liquid into excess acetone. Fraction C was redissolved[1] in 20 cc. of water and additional amounts of B_1 and B_2 fractions were separated as before. The process was repeated at an initial volume of 15 cc, and four times more at an initial volume of 10 cc. At the same volume Fraction C was separated twice at 66 per cent methyl alcohol concentration and again at 75 per cent, after which only traces of B_1 fractions remained. All B_1 fractions were combined, as were also the B_2 fractions.

Each of these three main fractions, C, B_2, and B_1, was further purified as follows:

Purification of Fraction C—The product was dissolved in 10 cc. of water, precipitated with 40 cc. of glacial acetic acid, and centrifuged off. The supernatant liquid was poured into 200 cc. of glacial acetic acid, yielding a precipitate (C_2). Fractionation of C with 80 per cent acetic acid was repeated twice, the last C_2 fraction being very small.

An aqueous solution of Fraction C was still quite turbid. It was accordingly diluted to 400 cc. and run twice successively through Berkefeld V, N, and W filters, and shaken with chloroform. The still opalescent solution was concentrated *in vacuo* to about 50 cc., centrifuged at 3000 R.P.M. for 8 hours to remove traces of insoluble material, concentrated *in vacuo* to small bulk, and poured into redistilled acetone. The product was washed three times with redistilled acetone and was filtered off and dried *in vacuo* over calcium chloride, paraffin, and NaOH pellets, a procedure followed with the other fractions as well. For analysis, this fraction, as well as all others, was dried to constant weight at 65° in a high vacuum over P_2O_5. An attempt to fractionate C with copper acetate followed by partial precipitation with methyl alcohol resulted in no marked change in the two principal fractions recovered except a partial concentration of the magnesium palmitate (see below) in the more soluble portion.

Purification of Fraction B_1—The combined B_1 fractions (see above) were dissolved in 5 cc. of water and precipitated with 20 cc. of glacial acetic acid. After the precipitate (B_1C) was centrifuged off, the supernatant liquid was poured into acetone,

yielding Fraction B_1. Fraction B_1C was reprecipitated as before. When Fraction B_1 was dissolved in a little water, crystalline magnesium phosphate remained. All additional inorganic phosphorus was eliminated by precipitating the carbohydrate five times with glacial acetic acid containing 15 per cent of redistilled alcohol[3] and washing with redistilled alcohol. The product was

TABLE I

Properties of Principal Polysaccharide Fractions of Human Strain (H-37) of Tubercle Bacillus

Fraction No.	Yield	$[\alpha]_D$	$[\alpha]_{Hg\ green}$	Neutral equivalent	Basic ash	N	P	Acetyl*	Pentose†
	gm.	degrees	degrees		per cent	per cent	per cent	per cent	
520, C	2.26	+85.2	+100.9	21,800	1.0‡	0.15	0.39	2.7	+±
CI		84.4	99.0	10,000	0.0	0.05	0.10	1.7	+±
C_2	0.32	72.0		2,100	2.0‡	0.62	1.90		
B_2	15.42	22.3	27.1	3,400	0.3‡	1.66	0.57	2.3	
" (alkali-treated)		22.8	26.7	3,100	0.0	0.44	0.07	3.5	+++
B_1	0.67	27.8	32.9	4,000	1.6‡	0.77	2.26	2.5	
B_2C	1.9	84.6	100.8	1,200	4.3‡	0.05	6.46		±
B_1C	0.7					0.06			±
									per cent
MB-200, CI		75.6		12,600	0.1§	0.01	0.13		20
B_2		23.8		2,700	0.0	0.62	0.53		37
B_1		62.0		1,000	2.3§	0.46	3.70		12

　* Hydrolysis with 42.5 per cent H_3PO_4.

　† Hydrolysis with 5 N H_2SO_4, orcinol test, and spectroscopic comparison of band intensities under controlled conditions.

　‡ Magnesium. $[\alpha]$, N, P, and acetyl calculated to ash-free basis.

　§ Calculated as Ca.

finally dissolved in a little water, centrifuged, and isolated by pouring into redistilled acetone.

Purification of Fraction B_2—The combined B_2 fractions (see above) were dissolved in 20 cc. of water and precipitated with 80 cc. of glacial acetic acid. After centrifugation, the supernatant liquid was poured into an equal volume of acetone, yielding Frac-

　[3] To prevent crystallization in the cold centrifuge.

tion B_2. The acetic acid-precipitable portion, B_2C, was again precipitated three times at 80 per cent acetic acid concentration, after solution in 10 cc., 5 cc., and 5 cc. of water respectively.   The fractions obtained on pouring the supernatant fluids into acetone were designated B_3 and B_4, the last two small fractions being combined.   Each of Fractions B_2 and B_3 was redissolved in water, treated with glacial acetic acid to incipient turbidity, centrifuged to remove the last traces of Fraction B_2C, precipitated with redistilled acetone, and isolated as in the case of Fraction C.

TABLE II

Properties of Subfractions from Acetic Acid-Precipitable Material of Human Strain (H-37) of Tubercle Bacillus

Fraction No.	Yield	$[\alpha]_D$	$[\alpha]_{Hg\ green}$	Neutral equivalent	Basic ash as Ca	N	P	Pentose
	gm.	*degrees*	*degrees*		*per cent*	*per cent*	*per cent*	
520, B_{2a}	0.48	+34.7	+40.5	5100	0.5	1.29	0.09	++
$B_{2a'}$	0.20	19.5	21.8	4500	0.2	1.28	0.15	
$B_{2a''}$	0.05	16.6	18.9	8600	0.7	1.22	0.10	
B_{2d}	0.08	13.9	16.8	5900	3.9	1.02	0.19	
B_{2b}	0.17	17*		4100				++
$B_{2b'}$	0.09	20.3	24.1	8000	2.0	1.65	0.88	
$B_{2b''}$	0.08	16*		3700				
B_{3a}	0.12	71.3		1600	3.6	0.18	3.41	
$B_{3a'}$	0.08							+±
B_{3b}	0.07	34.2		1400	5.6	0.65	5.93	
B_{3c}	1.23	23.7	29.8	4100	0.3	1.68	0.67	++
B_{3d}	0.42	22.4	27.9	2600	3.1	0.91	2.21	

* Not calculated to ash-free basis.

Fraction B_4 was reprecipitated with an excess of glacial acetic acid and isolated in the same way.   Fractions B_1C and B_2C were isolated after an additional precipitation with acetic acid by precipitating with acetone.   Both of these products were serologically inactive.

The chemical and physical properties of these fractions are summarized in Table I, the serological properties in Tables IV and V.   It was also possible to obtain very similar fractions, CI, B_2, and B_1, from the specific polysaccharide mixture isolated from

tuberculin (MB-200, *cf.* (4)).   Data on these fractions are also given in Tables I and IV.

It was found that Fraction B_2 from the cells was not serologically homogeneous in that it removed all carbohydrate antibodies from the test serum.   3 gm. were accordingly dissolved in 15 cc. of water and glacial acetic acid was added until flocculation occurred, 115 cc. being necessary.   The precipitate, B_{2a}, was centrifuged off and the supernatant liquid poured into 100 cc. more of glacial acetic acid, yielding another precipitate, B_{2b}.   The supernatant liquid from this was poured into an equal volume of acetone, yielding a third small fraction which was relatively inactive serologically.   All of these fractions were isolated as usual.

Fraction B_{2a}, about 1.7 gm., was dissolved in 3 cc. of water and methyl alcohol added until flocculation occurred, 18 cc. being necessary.   The precipitate, B_{2a}, was centrifuged off and the supernatant liquid poured into acetone, yielding Fraction B_{2d}.

1 gm. of Fraction B_{2a} was dissolved in 100 cc. of water and treated with 30 cc. of lead acetate solution.   There was no precipitation until after the addition of concentrated aqueous ammonia, which was added carefully until the first heavy flocculation occurred.   This was centrifuged off (Fraction B_{2a}) (see Table II) and to the supernatant liquid 2 cc. more of ammonia were added, giving another precipitate ($B_{2a'}$).   After addition of 7 cc. more of ammonia another fraction ($B_{2a''}$) was obtained.   The supernatant liquid was free from active material.   Fraction B_{2a} was freed from lead by solution in 3 cc. of 50 per cent acetic acid and precipitation with glacial acetic acid.   The resulting strongly turbid solution was flocculated by addition of a little glacial acetic acid saturated with sodium acetate.   The precipitate was dissolved in a little water and reprecipitated four times with acetic acid in the presence of sodium acetate, after which the supernatant liquid was free from lead.   To remove sodium acetate, the precipitate was washed with alcohol and reprecipitated from acetic acid a number of times, minimal amounts of LiCl being used to assist flocculation. The $B_{2a'}$ and $B_{2a''}$ precipitates were decomposed by CO_2 in the cold. After centrifuging and pouring off the supernatant liquids, the residues were treated again with water and CO_2 until practically free from material active with antiserum.   The combined supernatant liquids from each fraction were concentrated *in vacuo*,

precipitated with glacial acetic acid in the presence of sodium acetate, and isolated as in the preceding instances.

Most of Fraction B_{2d} separated with the initial addition of ammonia when a lead acetate fractionation was attempted. Analyses and serological tests were made on this portion.

Fraction B_{2b} was dissolved in 2 cc. of water and precipitated with 17 cc. of methyl alcohol (Fraction B_{2b}); by pouring the supernatant liquid into acetone Fraction $B_{2b'}$ was precipitated. By similar methyl alcohol fractionation, Fraction B_{2b} was resolved into Fractions B_{2b} and $B_{2b''}$.

Fractions B_3 and B_4 were combined and subjected to fractionation with glacial acetic acid and with methyl alcohol according to the methods described previously. The following subfractions were obtained: Fraction B_{3a}, precipitable at an acetic acid concentration of 81 per cent; on refractionation with acetic acid this fraction was resolved into Fraction B_{3a}, precipitable, and $B_{3a'}$, soluble at the same concentration. Additional subfractions, of which B_{3c} was the largest, were isolated from the initial 81 per cent acetic acid solution. These were Fraction B_{3b}, precipitable at a methyl alcohol concentration of 83 per cent; Fraction B_{3c}, soluble in 83 per cent methyl alcohol but precipitable with this reagent at 94 per cent; Fraction B_{3d}, soluble in 94 per cent methyl alcohol but precipitable with acetone.

Analytical and serological data on these fractions are reported in Tables II, IV, and V.

Carbohydrates Soluble in Strong Acetic Acid (Fraction II) (p. 80) —This fraction caused much difficulty owing to its high solubility in organic reagents and the presence of inorganic salts, especially phosphates. It was worked up essentially as follows: A portion precipitable with a larger excess of acetic acid (Fraction IIC) was removed and further fractionated, but as all fractions were relatively inactive serologically, their description is omitted. The supernatant liquid was concentrated to small bulk *in vacuo* and precipitated with 95 per cent ethyl alcohol (Fraction IIB). This fraction was about 5 times as large as Fraction IIC and yielded subfractions high in nitrogen but relatively inactive serologically. The vacuum-concentrated supernatant liquid from this yielded another portion precipitable with alcohol (Fraction III). Phosphate was removed from an aqueous solution of this fraction by

shaking with freshly precipitated $CuCO_3$, after which the supernatant liquid was taken down to small bulk *in vacuo* and brought to 75 per cent ethyl alcohol concentration. The supernatant liquid, which contained most of the serologically active material, was freed from traces of Cu^{++} with $K_4Fe(CN)_6$, concentrated to 5 cc., and fractionated with methyl alcohol. After removal of a small fraction precipitable with 6 volumes, a portion (Fraction III) was precipitated by an additional 10 volumes of methyl alcohol. The supernatant liquid yielded Fraction IIIa, the larger fraction, on being poured into acetone. The supernatant liquid from the crude Fraction III (above) was concentrated and again gave a precipitate with alcohol, Fraction IV. This was dissolved in 8 cc. of 50 per cent acetic acid containing a little LiCl and precipitated with 250 cc. of 95 per cent alcohol containing 30 cc. of acetone. After several fractional precipitations with methyl alcohol and acetone and a treatment with chloroform and octyl alcohol (12) the principal portion of Fraction IV was obtained soluble in 97 per cent methyl alcohol, in which it formed a brown solution. It was precipitated from this with acetone.

The supernatant liquids from Fraction IV were again concentrated *in vacuo* and precipitated with acetone, yielding Fraction V. This was worked up much as was Fraction IV, except that no appreciable fractionation was effected. The supernatant liquids from Fraction V were concentrated *in vacuo*, taken up in water, shaken with chloroform and octyl alcohol, concentrated again, and precipitated with absolute alcohol (Fraction VI). This was separated into a portion (Fraction VI) precipitated from glacial acetic acid by absolute alcohol, a fraction (VIa) thrown out from the supernatant liquid by acetone in the presence of LiCl, and Fraction VIb, isolated from the mother liquors with anhydrous ether. All of these fractions were highly active serologically. The supernatant liquid from the crude Fraction VI (above) was again concentrated *in vacuo* and precipitated with acetone (Fraction VII). This fraction, soluble in glacial acetic acid with a brown color, was isolated from 97 per cent methyl alcohol by precipitation with anhydrous ether.

The supernatant liquid from Fraction VII was taken down to dryness, dissolved in glacial acetic acid, and precipitated with ether. Redissolved in absolute alcohol, it was fractionally pre-

cipitated with ether, the first portion (Fraction VIII) requiring somewhat more than an equal volume of ether, Fraction VIIIa coming down with larger amounts.

The supernatant liquids from Fraction VIII (with some acetone supernatants from Fraction VII) were concentrated *in vacuo* and precipitated with anhydrous ether. The crude product (Fraction IX) was dialyzed in a cellophane tube against two changes of distilled water. The outside liquid contained salts and very little

TABLE III

Properties of Carbohydrate Fractions Soluble in Strong Acetic Acid

Fraction No.	Yield	$[\alpha]_D$	$[\alpha]_{Hg \ green}$	Neutral equiva-lent	Basic ash	N	P	Pentose
	gm.	*degrees*	*degrees*		*per cent*	*per cent*	*per cent*	.
520, III	0.33	+15.0		6100	1.5	5.26	0.29	
IIIa	0.67	21.7			4.0	5.50	0.90	
IV	3.88	21.0	24.3	1800	3.2	5.67	1.15	±
V	5.02	18.3	21.1		4.3	5.48	1.47	±
VI	0.15	39.6		3700	1.9	1.14	0.36	
VIa	0.13	25.0		3800	1.9	2.75	0.54	
VIb	0.07	43.5		6900	1.5	0.64	0.16	
VII	1.58	30.3		5200	3.5*	3.78	0.82	+
VIIa (from mother liquor)	0.36	15.2			5*	3.88	0.85	
VIII	0.75	9.5			13*	2.0	0.5	±
VIIIa	1.88	7			12*	1.2	0.3	±
IX	0.04	32			1.3*	2.4		
IXa†	0.10	35			1.3*	2.1		

* Calculated as Li + Ca. $[\alpha]$, N, and P calculated to ash-free basis.
† Reducing sugars on hydrolysis, 44 per cent.

serologically active material. The tube content was transferred to solution in absolute alcohol and separated into an ether-precipitable portion (Fraction IX) and a soluble fraction (No. IXa).

The properties of these fractions are summarized in Tables III, IV, and V.

Action of Sodium Hydroxide on Fractions

Fraction C—Since this product differed markedly in its chemical properties and appearance from corresponding fractions of earlier

preparations which had been purified by precipitation from alkaline solution by alcohol, about 1.3 gm. were dissolved in 100 cc. of N NaOH. A gelatinous mass slowly separated and was centrifuged off and washed three times with 10 cc. of N NaOH. The supernatant liquids were combined and treated with 25 cc. of a neutral copper acetate-tartrate solution prepared as follows: 21.3 gm. of tartaric acid were suspended in water and 16 cc. of saturated NaOH solution were added with stirring. The resulting hot solution was cooled and 100 cc. of saturated copper acetate solution were added, followed by 10 per cent NaOH until just alkaline to litmus. The volume was 170 cc. The specific polysaccharide was precipitated by this reagent (difference from Fraction B_2). After 2 hours in the cold, the precipitate was centrifuged off, dissolved in as little 50 per cent acetic acid as possible, and reprecipitated with excess acetic acid. The product was dissolved in a little water and again reprecipitated three times with glacial acetic acid, the usual washing with acetone and drying being omitted until the end.

The product (Fraction CI), which corresponded to Fraction C[4] (*cf.* (7)), was again reprecipitated, almost quantitatively, by bringing the concentration of acetic acid to 80 per cent, and was isolated in the usual manner. A small additional fraction (CI_a) settled from the copper supernatant liquid on long standing in the cold. After this was removed, the solution still contained small amounts of specific polysaccharide.

The insoluble product of the action of alkali on Fraction C was treated with N HCl, filtered, and washed with the acid and finally with water. The filtrate gave a positive reaction for Mg^{++} with Na_2HPO_4, NH_4Cl, and ammonia, and also contained traces of phosphorus.

The acid-insoluble material was dried *in vacuo*, dissolved in low boiling petroleum ether, filtered, and evaporated to dryness. The residue was taken up in 5 cc. of hot methyl alcohol, 3 cc. of hot water were added, and the solution was cooled slowly, first at room temperature, then in the ice box. The crystals which separated were taken up in 3 cc. of hot methyl alcohol and recrystallized by the addition of 1.5 cc. of hot water and slow cooling. After centrifuging and washing with 62 per cent methyl alcohol, the

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[4] Unsuccessful attempts were made to fractionate this material.

crystals were dried, finally over P_2O_5 in a high vacuum. Additional amounts were recovered from the mother liquors. The following data were obtained.

M. p., 53–57°; after recrystallization, m. p., 56–59°; mixed with highly purified palmitic acid (melting at 60.5–61.5°), fusion at 57–59°.

Analysis—$C_{15}H_{31}CO_2H$
        Calculated. C 74.92, H 12.59, neutral equivalent 256
        Found.   " 75.18, " 12.73,   "   "   257

For data on the polysaccharide component see Tables I, IV, and V.

Fraction B_2—Most of Fraction B_2 was subjected to an alkali-copper treatment like that for Fraction C. Although no appreciable precipitation occurred, ammonia was liberated. The polysaccharide was isolated as usual after removal of the copper by several glacial acetic acid precipitations.

Serological Properties of Principal Fractions (Tables IV and V)

For the qualitative serological tests 0.3 cc. of antiserum was mixed with 0.3 cc. of the carbohydrate dilutions in saline. For the cross-reactions 4 to 5 cc. of serum were repeatedly treated with about 0.05 mg. of a given fraction until a precipitate was no longer obtained after 48 hours. The absorbed serum was then set up as indicated in Tables IV and V. Readings were taken after the tubes had stood 2 hours at 37° and overnight in the ice box. Readings in parentheses in Table IV were taken after 5 to 10 minutes centrifugation at low speed. Although the principal active fractions gave positive tests at dilutions of 1:2 million and 1:4 million, comparisons in horse Serum 5807 are given at 1:500,000 in order to emphasize differences between active and inactive fractions. For the qualitative tests in rabbit antisera and the timothy bacillus horse serum dilutions of 1:50,000 were used.

The quantitative determinations were carried out according to previous descriptions (13–15). From 1 to 4 cc. of serum were used and all manipulations were carried out in the cold, the tubes being allowed to stand for 2 days after addition of polysaccharide. When quantitative cross-reactions were carried out, an aliquot portion of the supernatant liquid of the quantitatively absorbed

TABLE IV

Qualitative Tests of Carbohydrate Fractions in Whole and Absorbed Antisera

Fraction No.	Anti-H-37 Serum 5807, horse					Rabbit antisera			Horse antiserum
	Unabsorbed	Serum absorbed with fractions				H-37	Bovine	Avian†	Timothy
		C	CI	B₂	B₂d*				
520, C	+(+++±)	−(±)	−(−)	±(±)	++	+++	++	+++	±(++±)
CI	+	−(±)	−(−)	±(±)		+++			
B₂	+(+++±)	+±	+±	±(±)		+++			
" (alkali-treated)	+(++)	+±	++	±(±)					
B₁	+(+++±)				±(+±)	+++			
B₂C	−(−)				−(−)	−(−)			
B₁C	±(−)				−(−)	−(−)			
B₂a	+	++					±+		
B₂a'	++	+++				++	+	++	±±
B₂d	±+	++					++		
B₂b	+								
B₂b'	+±	+++±					±(+)	+++±	+(+++±)
B₃c	++						++		
B₃d	+(++)								
III	+(+)								
IV	+(+)								
V	±(+±)								
VI‡	±(+±)								
VII	−(+?)								
MB-200, CI	+±		−§	±(+++±)§		−(−)			±(∓±)
B₂	++		+++	±(±)					
B₁	±(++)		+++	−(±)					

Tests in the unabsorbed Anti-H-37 horse serum are given for 1:500,000 carbohydrate dilution. All other tests are for the 1:50,000 dilution. The usual controls were run. The parentheses denote readings after centrifugation.

* Similar results were obtained with B_{2a}' and weaker tests with B_{2b}'.

† Tests were run in antisera to avian S and avian R organisms but little difference was noted.

‡ The activity of all VI fractions was about the same. The VIII and IX fractions were negative at 1:500,000, but reacted at higher concentrations in Serum 5807.

§ In the tests with the MB fractions the serum was absorbed with the MB (tuberculin) fraction corresponding to that at the head of the column.

serum was set up with the second carbohydrate in the same manner. All quantitative determinations were carried out in duplicate. The values reported in Table V are mean values.

TABLE V

Antibody Nitrogen Precipitated by Carbohydrate Fractions from Antitubercle Bacillus Sera

Fraction No.	Amount used	Serum No.	Amount used	N pptd. per cc.	N pptd. per cc. original serum from supernatant by fractions			Total N pptd. per cc.
					C	B_{2b}''	B_{2a}	
	mg.		*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
520, C	0.30	5806, A	3.0	0.042				
CI	0.30	5806, A	3.0	0.038				
B_2 "	0.30	5806, A	3.0	0.044				
(alkali-treated)	0.30	5806, A	3.0	0.039				
C	0.15	5807, A	1.0	0.12				
"	0.20	5807	2.0	0.10		0.01_5		0.11_5
CI	0.20	5807	2.0	0.09		0.02_5		0.11_5
B_{2a}	0.20	5807	2.0	0.12				0.12
B_{2a}'	0.20	5807	2.0	0.09	0.01			0.10
B_{2b}'	0.20	5807	2.0	0.11	0.00_5			0.11_5
B_{3d}	0.20	5807	2.0	0.10	0.01_5			0.11_5
VII	1.6	5807	4.0	0.071	0.03	0.03	0.04	0.11*
VIII	0.8	5807	2.0	0.06			0.04	0.10
VIIIa	1.6	5807	4.0	0.038	$0 06_5$	0.07	0.08	0.12*
IX	0.40	5807, L†	2.0	0.03			0.06_5	0.09_5
IXa	0.40	5807, L†	2.0	0.02			0.07_5	0.09_5
C	0.20	5807, L†	2.0	0.09				

Rabbit Anti-H-37 sera

C	0.10	10.71_1	1.0	0.09				
"	0.20	2.34	3.0	0.075				
CI	0.20	2.34	3.0	0.06				
B_2	0.10	10.71_1	1.0	0.07				
"	0.075	10.69	1.0	0.05				

* N precipitated by Fraction B_{2a} is used in these cases in computing total N.

† Lyophile-dried, redissolved in water.

Hydrolysis of Specific Polysaccharides

Four different fractions of earlier preparations were hydrolyzed. Two of these fractions corresponded about to Fraction 520,B_{2a}.

The fraction of which the hydrolysis is given first approximated Fraction 520,B_{2b}, while the fourth corresponded to Fraction CI. All carbohydrates hydrolyzed were prepared with the aid of alkali. From all except Fraction CI d-arabinose was isolated directly in crystalline form. This sugar was also present in Fraction CI, but it could be isolated only as the diphenylhydrazone. In every instance except the first, d-mannose was found among the hydrolysis products and was identified as the phenylhydrazone. Attempts to isolate mannose as such failed.

(a) 2.66 gm. of Fraction 518,B_1 (7) were hydrolyzed with 150 cc. of boiling N H_2SO_4. The specific rotation dropped from $+24.2°$ to $-23.6°$ after 1 hour and to $-22.0°$ after 2 hours, at which times the reducing sugar content (Shaffer-Hartmann) was 63.1 and 64.3 per cent. The liquid was cooled and neutralized with barium hydroxide. The supernatant liquid was concentrated in $vacuo$ and treated with absolute alcohol. The resulting gummy precipitate was extracted three times with boiling absolute alcohol and the extracts were combined with the first alcoholic supernatant liquid. The alcohol-insoluble residue was extracted four times with boiling methyl alcohol, the extracts, concentrated in $vacuo$, yielding 17.2 mg. of a phenylosazone melting at 160–165° and decomposing at 200°.

The combined absolute alcohol extracts were concentrated, freed from a slight deposit by centrifugation, and seeded with a few crystals of d-arabinose. The first crop of the pentose was recrystallized twice from dilute alcohol, with a little norit, and washed with 70 per cent and absolute alcohol. Yield 15 mg.; $[\alpha]_D$, initial, $-189°$; final, $-116°$. The filtrates from the second recrystallization of this crop were chilled and seeded with a few crystals of the original fraction. After 2 days standing in the cold a larger portion of d-arabinose was collected and washed with a little 95 per cent alcohol. The yield was 44 mg.; $[\alpha]_D$, initial, $-160°$; final, $-106°$. Both crops were combined, dissolved in a little water, treated with 100 mg. of sodium acetate, 100 mg. of freshly recrystallized diphenylhydrazine hydrochloride, and enough alcohol to clear the solution. This was kept first at room temperature and then in the cold. The resulting diphenylhydrazone was recrystallized from 95 per cent alcohol. Yield 92.6 mg.; m.p. 203°; $[\alpha]_D$ in pyridine-alcohol (3:2) $-15.2°$.

Analysis—(Micro-Dumas). $C_{17}H_{20}O_4N_2$. Calculated. N 8.86
                                                            Found.   " 8.77

The filtrates from the first recrystallization of the first crop of
d-arabinose were combined with the filtrate from the second crop,
concentrated, and treated at a volume of about 5 cc. with a mixture
of 1 cc. of glacial acetic acid and 1 cc. of freshly distilled phenyl-
hydrazine at room temperature.   The resulting yellow precipitate,
which appeared to be an osazone, was recrystallized from 10 per
cent alcohol.   Yield 74.4 mg.; m.p. 160–162° with preliminary
softening; $[\alpha]_D$, initial, $-39°$; final, $-23.5°$, in pyridine-alcohol.
Levene and La Forge (16) give the initial and final rotation of
l-arabinosazone as $+55°$ and $+30°$.   According to Weerman (17)
d-arabinose phenylosazone melts at 161–162°, so that the above
product would seem to be this substance in somewhat impure
form.

The original supernatant liquid from the first crop of *d*-arabinose
was concentrated to small bulk and placed in the ice box.   After
several days the crystalline deposit was recrystallized from dilute
alcohol and washed with 70 per cent alcohol and again recrystal-
lized as before.   The supernatant liquid and washings were
combined with the original mother liquor.   Yield 57 mg.; $[\alpha]_D$,
initial, $-151°$; final, $-102.7°$.   The fraction was evidently chiefly
d-arabinose.   From the supernatant liquids additional crystals
were obtained.   These contained *d*-arabinose and another sugar
which was not identified, since only derivatives characteristic of
d-arabinose could be isolated from the mixture.   No evidence for
the presence of mannose could be obtained.

(*b*) 2.4 gm. of Fraction 519,B_8 were hydrolyzed in the same
way.   After removal of the H_2SO_4 the solution was concentrated
in vacuo and treated with 2 volumes of absolute alcohol.   The
brown precipitate which formed was not further examined.   The
supernatant liquid was treated with CO_2 to remove traces of Ba^{++},
concentrated to a syrup, and precipitated with absolute alcohol.
The supernatant liquid from this was freed from alcohol, diluted,
treated with norit, filtered, and concentrated.   Alcohol was
added and the crystalline deposit filtered off.   On repeated
crystallization from dilute alcohol these fractions yielded 0.37 gm.
of pure *d*-arabinose.   The portion analyzed melted at 155–158°
with preliminary softening. $[\alpha]_D$, initial, was $-156°$; final,

−105°; compared with the values given in the literature (18), m.p. 156–157°, and $[\alpha]_D$ −175°, −105° respectively. Calculated for $C_5H_{10}O_5$, C 40.18, H 6.78 per cent; found, C 39.98, H 6.72 per cent.

In this case the supernatant liquids from the d-arabinose gave d-mannose phenylhydrazone with phenylhydrazine and acetic acid in 0.31 gm. yield. Recrystallized from 60 per cent alcohol, the hydrazone melted and decomposed at 198.5–199.5° with preliminary darkening. $[\alpha]_D$, in pyridine-alcohol, initial, +15.8°; final, 0°. The values given by Hoffmann (19) are m.p. 199–201°, $[\alpha]_D$ +26.6° in pyridine with no mutarotation. The filtrates from the hydrazone, freed from phenylhydrazine with benzaldehyde, gave 0.40 gm. of d-arabinose diphenylhydrazone with diphenyl-hydrazine hydrochloride and sodium acetate. Recrystallized from 95 per cent alcohol, this was recovered in two portions, melting with decomposition at 202–204° and 203–204.5°; $[\alpha]_D$, in pyridine-alcohol, initial, −27.8°, −20.8° respectively; final, −22.2°, −18.2°. No additional components of this fraction could be identified.

Analogous results were obtained on hydrolysis of a similar fraction.

(c) Hydrolysis of about 1 gm. of Fraction 519,CI under similar conditions required 7 hours. The initial $[\alpha]_D$, +79°, dropped to −16°. The hydrolysis mixture was worked up as before, but the component sugars could be isolated only as their hydrazones. With phenylhydrazine and acetic acid in the cold d-mannose phenylhydrazone was obtained, melting at 197–198° with decomposition and with preliminary darkening after two recrystalliza-tions from 60 per cent alcohol. $[\alpha]_D$, in pyridine-alcohol, initial, +25°; final, +14°. Yield 0.27 gm.

Analysis—(Micro-Dumas). $C_{19}H_{16}O_5N_2$. Calculated. N 10.45
                                                     Found.   " 10.40

Another lot of 40 mg., melting at 195–197°, was recovered from the concentrated mother liquors. After elimination of excess phenylhydrazine with benzaldehyde and removal of the latter with butyl alcohol the mother liquors were treated in the usual way, yielding d-arabinose diphenylhydrazone. After two re-crystallizations from alcohol the substance melted at 204–205°; $[\alpha]_D$, initial, −13°; final, −26°.

Analysis—(Micro-Dumas). $C_{17}H_{20}O_4N_2$. Calculated. N 8.86
Found.   9.14

DISCUSSION

From the experimental data it is evident that the material of carbohydrate nature which can be extracted without the use of heat, alkali, or strong acid from the dried, defatted cells of a human strain, H-37, of the tubercle bacillus, is an extremely complex mixture of serologically active and inactive polysaccharides. In addition to glycogen, which had already been found by Laidlaw and Dudley (1), there is at least one other relatively acidic inactive polysaccharide present in Fractions B_1C, B_2C, IIB, and IIC. The bulk of the serologically active polysaccharides may be separated into a strongly dextrorotatory fraction (C), relatively insoluble in strong acetic acid and in 75 per cent methyl alcohol, and about 7 times as much of a soluble, weakly dextrorotatory fraction (B_2), each showing characteristic chemical differences. Of the large number of fractions separable by a variety of methods from the B_2 mixture, three were obtained, namely Fractions $B_{2a''}$, $B_{2b''}$, and B_{2d}, which possessed a serological specificity distinct from Fraction C in that they not only gave precipitates in Anti-H-37 horse serum which had been absorbed with Fraction C, but, when used to absorb other portions of the same antiserum, also failed to remove all antibodies for Fraction C. These B fractions were, however, comparatively small, and most of the low rotating material ultimately remained in fractions which appeared to possess both specificities, so that they possibly consisted mainly of a third specific polysaccharide combining the immunologically active groupings of the other two. This view appears more probable than the alternative of a mixture of the C and $B_{2a''}$ polysaccharides, since the copper acetate-tartrate reagent used precipitated the C (CI) fraction but failed to throw down any of the B_2 material. As stated before, the solubilities were also very different. However, the quantitative precipitin tests (Table V) showed a certain overlapping of specificity for even the C and $B_{2a''}$ fractions, so that a more definite interpretation of the findings can scarcely be given. Even the end-fractions, with their unusual solubility in absolute alcohol and acetone, appeared to react with a portion of the same antibodies

(Table V), so that it is possible that these fractions consisted of one or more of the others with a portion of their reactive groupings covered or sterically hindered by chemical groupings enhancing solubility in alcohol and acetone. Strangely enough, Fraction IXa, which was not precipitable by ether, yielded almost 50 per cent of reducing sugars on hydrolysis. Unfortunately the only antisera available were exceedingly weak, containing only 5 to 10 per cent as much antibody as is present, for example, in a good antipneumococcus serum. Thus it is possible that greater differences might have been found in the behavior of the fractions in more potent antisera.

None of the polysaccharide fractions tested appeared to be type-specific, since they reacted in antisera to the bovine and avian types of the tubercle bacillus and to the timothy grass bacillus (Table IV).

In contrast to the behavior of the specific polysaccharide of Type I pneumococcus (20) the immunological properties of the tubercle bacillus fractions did not appear to be changed by the action of alkali, although the chemical properties of one fraction (C) underwent a very marked change. When made alkaline, the opalescent aqueous solution of this fraction slowly deposited a gelatinous precipitate consisting largely of magnesium palmitate and yielded a polysaccharide (CI) soluble in water to form clear solutions and apparently identical with earlier C fractions, in the isolation of which alkali had been used. The CI fraction·precipitated as much antibody from Anti-H-37 horse serum as did the C fraction, contrary to the behavior of the Type I pneumococcus polysaccharide (20, 21), which loses an acetyl group with alkali.[5] While it is possible that the palmitic acid in the C fraction originated in a lipid impurity, its presence is suggestive of the carbohydrate-lipid complexes isolated by Landsteiner and Levene from the Forssman hapten (22), and by Boivin and his collaborators (23) and Raistrick and Topley (24) from the salmonella group. These substances, however, were antigenic in rabbits, while the C fraction failed to produce precipitins when injected intravenously in varying amounts into three rabbits. The more

[5] A difference was, however, noted in a rabbit Anti-H-37 serum, recalling the greater sensitivity of rabbit antipneumococcus sera to degradative changes in pneumococcus polysaccharides (21). See Table V.

sensitive complement fixation test, however, gave indications of antibody formation in one rabbit.

While the work in hand concerned itself principally with the specific polysaccharides of the tubercle bacillus cell, a comparison was made with the polysaccharides of tuberculin. For this purpose a preparation, MB-200, isolated by Masucci, McAlpine, and Glenn (4), was kindly furnished by these workers. It was found to contain the same principal components as the cellular material, being separable into three fractions corresponding to Fractions CI, B_1, and B_2 (Tables I and IV). Of these, Fractions CI and B_2 were immunologically distinct without further fractionation.

Hydrolysis of the principal fractions of the cell polysaccharides by means of mineral acid showed them to consist mainly of d-arabinose and d-mannose in varying proportions, two sugars which have not been reported in the specific polysaccharides of other microorganisms. d-Arabinose, while a common laboratory product, occurs only rarely in nature, as, for example, in barbaloin (25). In the present work the sugar was isolated from tubercle bacillus polysaccharides for the first time in crystalline form (7), although "arabinose" diphenylhydrazone had been obtained previously from such material by Dorset and Henley (5) and d-arabinose benzylphenylhydrazone had been identified by Renfrew (3) and Chargaff and Anderson (8). While it was possible to isolate crystalline d-arabinose from all three B fractions hydrolyzed, these being high in pentose content, the sugar could be identified only as the diphenylhydrazone in a CI fraction, in which it occurred in smaller amount. The mannose present could not be obtained crystalline, and was identified in two of the B fractions and the CI fraction as the phenylhydrazone. It could not be found in one of the B fractions (see the experimental part). Small amounts of other sugars and acid components were present in the fractions, but these were not identified. Several fractions were tested for inosite with negative results (cf. (8, 9)).

Since the neutral equivalent of the C fractions is 10,000 and above, and the serologically inactive fractions are the most strongly acidic, it would appear that sugar acids have little connection with the immunological specificity of the polysaccharides of the tubercle bacillus group.

SUMMARY

1. The polysaccharide portion of tubercle bacillus cells of a human strain, H-37, has been obtained as a complex mixture of serologically active and inactive polysaccharides. Fractionation of the mixture is described.

2. One of the two serologically distinct specific polysaccharides isolated is relatively highly dextrorotatory, low in pentose, and appears to contain magnesium palmitate in chemical combination.

3. The other serologically distinct polysaccharide has a low dextrorotation and is difficultly separable from larger amounts of similar material combining both specificities, and, in general, showing relatively high pentose content.

4. The fractions are built up chiefly of d-arabinose and d-mannose units in varying proportions.

5. Analogous fractions are contained in the polysaccharides of tuberculin.

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THE CYSTINE CONTENT OF INSULIN

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That cystine is present in crystalline insulin has been shown beyond any question by the actual isolation of this amino acid from the hydrolyzed crystalline insulin (1). The actual amount of cystine, however, has been a matter of some speculation. The question has been whether cystine can account for the entire sulfur content of insulin or whether there is present some other sulfur-containing moiety. With all the work which has indicated the close relationship between the sulfur and the physiological activity of insulin, the importance of settling this question is apparent.

The possibility of the presence of methionine and thiolhistidine has been investigated but the evidence available has indicated their absence. Freudenberg, Dirscherl, and Eyer (2) could find no methylthiol grouping in crystalline insulin, an observation which has been confirmed by Jensen and coworkers (3) on both hydrolyzed and unhydrolyzed insulin. With regard to thiolhistidine, the work of du Vigneaud, Sifferd, and Miller (4) has demonstrated the lack of this amino acid in insulin. This observation has also been confirmed by Jensen and coworkers (3) who used a different method.

On the basis of the Sullivan method which has been shown to be highly specific for cystine, only approximately three-fourths of the sulfur has been accounted for as cystine. A value of 8.3 per cent cystine in the crystalline insulin was reported by du Vigneaud, Jensen, and Wintersteiner (1), whereas, on the basis of the sulfur content of the sample of insulin used, approximately 11.8 per cent cystine could have been present if all of the sulfur had been due to this amino acid.

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Among the possibilities which occurred to us, which might explain the difference between the Sullivan values and the total sulfur, was the possibility that in the hydrolysis of insulin a difficultly hydrolyzable cystine peptide might have remained which did not respond to the Sullivan method.   In a few earlier experiments which will not be reported in detail here because of their essentially negative results with regard to the present question, insulin was first treated with crystalline pepsin prepared by Northrop's method (5) and then subjected to acid hydrolysis.   It was hoped that certain linkages might be split by the enzymatic hydrolysis which would render the resulting material more susceptible to acid hydrolysis and that a higher cystine value might thus result.   When no higher cystine content was indicated by this approach, some other method of hydrolysis was sought.

A method of hydrolysis which seemed worth applying was that used by Gurin and Clarke (6) for the hydrolysis of peptides in which 50 per cent formic acid with 2 equivalents of HCl for each equivalent of nitrogen was employed.   Blumenthal and Clarke (7) also used this method for the hydrolysis of proteins, refluxing the mixture for 16 hours.   In applying the method to insulin, however, we found that after 16 hours of refluxing the mixture yielded a practically negative Sullivan reaction.   The hydrolysate, of course, was evaporated to dryness in order to get rid of the formic acid before applying the reaction.   Even when the duration of hydrolysis was extended to 48 hours, less than 4 per cent of cystine was found by the Sullivan method.   Experiments were then carried out in which the amount of HCl was increased to 20 per cent HCl in the 50 per cent formic acid and crystalline insulin was hydrolyzed with this mixture.   The experiments revealed that hydrolysates prepared with this $HCl-HCO_2H$ mixture consistently yielded higher cystine values than did those prepared with HCl alone.   Moreover, it was found that the amount was augmented as the period of refluxing was increased.   Simultaneously with the Sullivan determinations, analyses for cystine were made by the Folin-Marenzi method.   As the time of hydrolysis was lengthened, the Folin-Marenzi cystine values at first rose much more rapidly than the corresponding Sullivan values but subsequently decreased until the values were identical to those given by the

Sullivan method. This phenomenon is similar to that reported by Jones and Gersdorff (8) on the difference between the results with these two methods in a study of the rate of liberation of cystine during the acid hydrolysis of casein. The most striking point with insulin was that the cystine content which we obtained by the Sullivan method gradually increased with increasing length of hydrolysis until at 48 hours it agreed with the value theoretically possible on the basis of the sulfur content of the insulin. This was repeatedly confirmed. Further hydrolysis did not change the value appreciably. Experiments were then devised to ascertain to what factors the higher results were due and
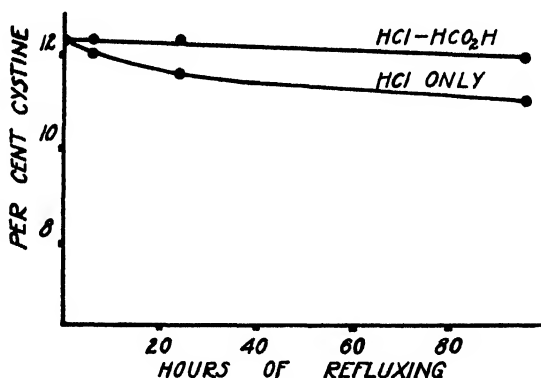
Fig. 1. Rate of destruction of cystine when refluxed with HCl and with HCl-HCO$_2$H in the presence of a mixture of amino acids.

whether the values so obtained could be relied upon as indicating the true cystine content. We wished to make certain that the results were not due to a fortuitous agreement between the cystine and sulfur contents.

In order to find out whether cystine could be determined accurately when it was refluxed for varying periods of time with an HCl-HCO$_2$H mixture in the presence of other amino acids known to occur in insulin, a mixture of amino acids containing cystine was made up and was treated in a manner similar to the procedure employed for the hydrolysis of insulin. Comparable experiments were carried out with 20 per cent HCl. The results shown in Fig. 1 indicate that when this mixture was refluxed with the HCl

alone, a gradual decrease in the cystine content occurred, whereas with the HCl-HCO$_2$H mixture, practically no change took place. The results indicated, therefore, that the cystine could be accurately determined when the mixture had been refluxed with HCl-HCO$_2$H and that apparently the HCO$_2$H protected the cystine from destruction. It is of interest to recall that Andrews (9) has demonstrated that cystine when refluxed with HCl is oxidized to cysteic acid by atmospheric oxygen. No such effect was found by Andrews with H$_2$SO$_4$. It might be pointed out, however, that H$_2$SO$_4$ with insulin yields considerable humin. HCl yields a somewhat yellow hydrolysate with slight humin formation, but the HCl-HCO$_2$H hydrolysis of insulin gave no indication whatsoever of humin formation.

One point further which needed investigation was whether the HCl-HCO$_2$H treatment might bring about some reduction of cystine to cysteine and in this way produce an augmented colorimetric value and mask an actual destruction. However, the hydrolysates gave no color with the sodium nitroprusside reagent nor did the Okuda method (10) indicate the presence of any cysteine.

From the results of these experiments with the synthetic amino acid mixture, we would conclude that the reason for the lower Sullivan values in previous investigations of insulin, where HCl alone was used, was due to slow liberation of cystine towards the end of the hydrolysis, on the one hand, and destruction of cystine, on the other. A maximum value was, therefore, reached rather early in the hydrolysis. That this apparent maximum was to be explained on this fortuitous balanced liberation and destruction of cystine was verified by applying the HCl-HCO$_2$H hydrolysis to a sample of insulin which had already been hydrolyzed by 20 per cent HCl for 24 hours. The cystine value was shown to be increased, whereas controls with HCl alone showed no appreciable change during the second 24 hour period.

Further experiments were then undertaken to show the rate of liberation of the cystine throughout the period of hydrolysis by the HCl-HCO$_2$H with both the Folin-Marenzi and Sullivan methods. Companion experiments were also run with just 20 per cent HCl. The results are shown in Fig. 2.

We should like to point out that the apparent variability of the

sulfur content of insulin occasionally reported might well be due
to the variation in moisture content.   We have found that insulin
is far more hygroscopic than generally recognized.   Air-dried
insulin may contain as much as 12 per cent moisture.   The method
of determination makes considerable difference.   We have encoun-
tered the fact that with the ordinary Fischer vacuum dryer in
which P_2O_5 is used, the insulin comes apparently to a constant
weight, but, when the sample is placed in a modified Pregl micro-
desiccator in which a slow stream of dry air passes over the sample,
a greater loss of moisture results.   Such a dried sample is ex-
tremely hygroscopic, and special care is required so that the dried
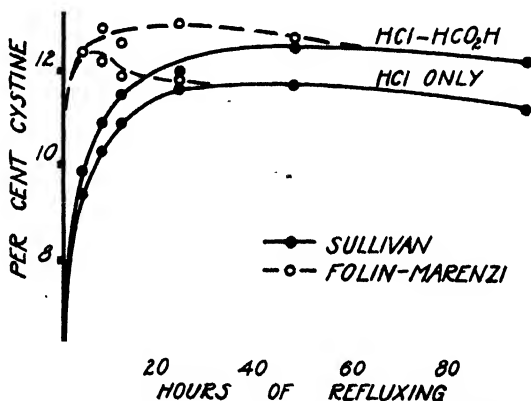
Fig. 2. Rate of liberation of cystine in the hydrolysis of crystalline
insulin with HCl and with $HCl-HCO_2H$.

sample does not come in contact with air before or during the
weighing.   With such a factor playing a rôle it is no wonder that
the sulfur content will be found to vary, depending on the history
of the compound and method of determination of the moisture.
Whether a true variation occurs in the sulfur content is another
matter upon which our evidence does not bear.   We have not
encountered a variation such as reported by various investigators.
The values of cystine reported here are based on a moisture-free,
ash-free basis.   Upon this basis, the sulfur content was found to be
3.34 ± 0.03 per cent and the cystine content 12.5 ± 0.4 per cent,
by the $HCl-HCO_2H$ hydrolysis.   The latter value agrees with
the theoretically possible cystine content.

For this investigation 4.5 gm. of beef insulin[1] containing 18 units to the mg. were crystallized by Scott's procedure (11). The yield was 50 per cent. This product, however, possessed a relatively high ash content. By recrystallization from acetic acid, pyridine, and ammonia according to the method of du Vigneaud, Jensen, and Wintersteiner (1) the ash content was reduced below 1 per cent. The yield on recrystallization was 80 per cent.

The moisture content of the air-dried crystals was first determined with the aid of a Fischer vacuum dryer at 114° with P_2O_5 as the drying agent. The method yielded inconsistent values and, as will be seen later, did not give the true moisture content. It became apparent that crystalline insulin is very difficult to dry and, furthermore, that the dried material absorbs moisture from the atmosphere extremely readily. A modified Pregl microdesiccator (12) proved more efficient than the Fischer dryer. A drying pig was employed for handling the samples, so that they did not come more than momentarily in contact with the air. It was found that by this method the insulin could be brought more readily to a constant weight and consistent values could be obtained on a given sample. As representative of the data which we have obtained, the following example is given. Samples of air-dried crystals when dried in the Fischer dryer at 25 mm. pressure yielded the moisture values 6.4, 5.8, and 6.8 per cent respectively. When the air-dried crystals were placed in an ordinary vacuum desiccator at 30 mm. over P_2O_5 for 5 days, they lost 5.2 per cent moisture. Duplicate samples of the desiccator-dried crystals were then placed in the microdesiccator at 5 mm. pressure. Each sample reached a constant weight which indicated a further loss of 5.0 per cent moisture. The total moisture of the air-dried crystals was, therefore, 10.2 per cent. This moisture value was confirmed by repeated analyses.

When analyzed for sulfur, samples of the air-dried crystals of known moisture content yielded the values 3.00, 2.98, 3.00, and 2.95 per cent, the average of which is 2.98 per cent. The ash content of these air-dried crystals was 0.6 per cent. Therefore,

on a moisture-free, ash-free basis the sample of crystalline insulin contained 3.34 ±0.03 per cent sulfur, which could represent 12.5 ±0.1 per cent cystine if all of the sulfur were present in this form.

Colorimetric Methods Employed—The Sullivan method was applied as described by Sullivan and Hess for protein analysis (13), except that 1 per cent sodium-β-naphthoquinone-4-sulfonate was used. The larger amount of dye reagent, which has been resorted to in certain instances by Sullivan, was used to offset any possible inhibitory effect of substances in the insulin hydrolysates which might react with the dye. On the basis of the work of Bushill, Lampitt, and Baker (14) on the proportionality between the extinction coefficient and the cystine content in the Sullivan method, a green filter (Wratten No. 58) was employed for the color comparisons. The green filter also noticeably relieved eye fatigue. Duplicate determinations of unknown solutions agreed within 3 per cent. For the determinations by the Folin-Marenzi method (15) a cystine standard was made up of 1 cc. of 0.5 N H_2SO_4 containing 0.4 mg. of cystine, to which were added 2 cc. of water. The unknown solution was prepared by adding 1 cc. of 0.5 N H_2SO_4 to a suitable aliquot of the hydrolysate (prepared as above and adjusted to pH 3.5) and then adding enough water to give 3 cc. total volume. To the standard and the unknown were added in succession the following reagents: (1) 1 cc. of 10 per cent Na_2SO_3, (2) after a 1 minute interval, 5 cc. of 18 per cent Na_2CO_3, (3) 1 cc. of 10 per cent Li_2SO_4, (4) 2 cc. of phosphotungstic acid reagent, (5) after an interval of 5 minutes, 13 cc. of 3 per cent Na_2SO_3. Duplicate determinations of samples from hydrolysates agreed within 3 per cent.

In order to ascertain as far as possible the effect on the color reagents of products of insulin hydrolysis other than cystine, a "synthetic" insulin hydrolysate, described later, was compared by both colorimetric methods with a standard solution containing an equivalent amount of cystine. It was found that in neither method did the other amino acids have a detectable influence on the extent of color development.

Experiments with Amino Acid Mixtures—An amino acid mixture containing 13.3 mg. of arginine, 18.3 mg. of histidine, 9.5 mg. of lysine, 50 mg. of tyrosine, 83 mg. of glutamic acid, 125 mg. of leucine, 48 mg. of cystine, 21 mg. of phenylalanine, and 21 mg. of

proline was prepared. Except for the amounts of the three amino acids last mentioned, which were arbitrary estimates, the amounts of amino acids used in the mixture were approximately in the proportion known to be present in insulin to the best of our knowledge (1, 16–18). The mixture was dissolved in 60 cc. of concentrated HCl. Two 29 cc. portions were pipetted into separate flasks. To one portion were added 31 cc. of water, to the other, 31 cc. of constant boiling HCO_2H. Three 12 cc. aliquots of each of these final mixtures were refluxed for the time periods 6, 24, and 96 hours respectively. Also, one 12 cc. aliquot of each type of solution was evaporated directly to dryness and was used as a blank. The solutions after being refluxed were evaporated to dryness. The residues were made up to 10 cc. at pH 3.5 and the cystine content of the solutions thus obtained was determined by the Sullivan method. The results are shown in Fig. 1.

Cystine Content and Rate of Liberation of Cystine in Hydrolysis of Insulin—Air-dried crystals of known moisture content were used for the hydrolysis experiments. Approximately 40 mg. samples were weighed directly in the tubes in which the hydrolyses were to be carried out. The samples were first dissolved in 5.8 cc. of concentrated HCl and then these solutions were diluted with 6.2 cc. of water for the HCl hydrolyses or with constant boiling HCO_2H for the HCl-HCO_2H hydrolyses. The 20 per cent HCl solutions of insulin were rather cloudy, whereas the HCl-HCO_2H solutions were perfectly clear. The hydrolysis tubes were then fitted by ground glass joints to air-cooled condensers and the tops of the condensers were fitted with soda-lime tubes. The solutions were heated at 110–120° for the periods of 4, 8, 12, 24, 48, and 96 hours, a separate 40 mg. sample being used for each time period. At the end of the desired periods of hydrolysis, the solutions, protected from contamination, were evaporated to dryness before a fan and the residues were made up to 10 cc. of solution at pH 3.5. The colorimetric methods were then applied as described above to aliquots of these final solutions. Duplicate determinations with each of the methods were carried out in all instances. The results showing the rate of liberation of the cystine are given in Fig. 2.

The authors wish to thank Mr. C. Rodden, microanalyst of this laboratory, for carrying out the microanalyses.

SUMMARY

It has been shown that the sulfur of insulin can be entirely accounted for as cystine on the basis of the Sullivan method within the experimental error of the method. Not more than traces of other sulfur compounds can be present. The cystine content of crystalline insulin containing 3.34 ± 0.03 per cent sulfur on a moisture-free, ash-free basis was shown to be 12.5 ± 0.4 per cent.

The lower values by the Sullivan method obtained heretofore, namely 8 to 9 per cent, were shown to be most likely due to incomplete hydrolysis on the one hand and destruction of the cystine during the hydrolysis on the other.

In the present investigation the hydrolyzing agent employed was 20 per cent HCl in 50 per cent formic acid based on the HCl-HCO_2H procedure of Gurin and Clarke. The cystine was apparently protected from destruction during the hydrolysis.

Addendum—During the preparation of this manuscript we received from Dr. Erwin Brand a copy of a forthcoming note in the *Proceedings of the Society for Experimental Biology and Medicine* in which Kassell and Brand reported finding 0.7 per cent of methionine in a sample of crystalline insulin and that the remainder of the sulfur was present as cystine. Dr. Brand submitted the manuscript to us, knowing that we had been engaged for some time in the study of the cystine content of insulin, and we should like to take this opportunity of expressing our appreciation of this courtesy on his part. Such a trace may or may not be present so far as our experimental data obtained with the Sullivan method are concerned.

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THE PHOSPHOLIPID METABOLISM OF TUMORS*

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The presence of large amounts of phospholipid in tumors of various kinds has been shown by Bullock and Cramer (1914), Lewis (1927), Jowett (1931), Yasuda and Bloor (1932), Bierich, Detzel, and Lang (1931), and Bierich and Lang (1933). Moreover the work of Jowett (1931), Bierich, Detzel, and Lang (1931), and Yasuda and Bloor (1932) proves that malignant tumors contain larger amounts of both phospholipid and cholesterol than benign tumors. The significance of these results has been discussed at length by various workers. Beard (1935) states that "it seems reasonable to believe that the cancer cell may utilize fat as a source of energy for its rapid growth." Lewis (1927) discusses the importance of the phospholipid and cholesterol content of cancer cells from a physicochemical standpoint. Rondoni (1930) and Tesauro (1932) maintain that tumor growth is promoted by cholesterol and retarded by phospholipids. On the other hand, Lemay (1931), Jowett (1931), and Bierich, Detzel, and Lang (1931) attribute tumor growth promotion to phospholipids and its retardation to cholesterol.

It is difficult to arrive at any conclusion concerning the importance of the phospholipids in cancer cells as long as their function in normal cells remains obscure. There are three theories regarding the function of phospholipids in normal cells; namely, those of fat metabolism, of oxygen transport, and of cellular structure (Sinclair, 1934; Artom, 1935). Recently Sinclair (1935, b) has shown that at least two classes of phospholipid may exist:

one, the metabolic, functioning as an intermediate in fat metabolism; the other, the non-metabolic, functioning as an essential part of the cellular structure.   Liver has been shown by him to contain both classes of phospholipid, while muscle contains mainly non-metabolic phospholipid.   This conclusion was reached by feeding elaidin and showing that elaidic acid replaces part of the normal phospholipid fatty acids of the rat, and that its entrance into and disappearance from the phospholipids is rapid in liver but comparatively slow in muscle.

The present paper is a similar study of tumor phospholipids made by feeding elaidin to rats inoculated with a transplantable tumor.   In addition to determining the rate of entrance into and disappearance of the elaidic acid from the tumor phospholipids, we wished to obtain information concerning the ratio of solid to liquid fatty acids of the phospholipids as a possible explanation of their low degree of unsaturation (Haven, 1935).

EXPERIMENTAL

Tumor—The tumor used throughout the following experiments was rat Carcinosarcoma 256 obtained from the Institute of Cancer Research, Columbia University.   Tumor tissue was inoculated by means of a trochar into the groin of young male rats weighing from 70 to 100 gm.

Analytical Methods

Samples of tissue to be analyzed were always taken from the outside or growing portion of the tumors, since this tissue has been shown to contain more phospholipid than the center or non-growing portion of the tumors (Haven, 1937).   The micro-fractionation of the phospholipid fatty acids was carried out by the method of Sinclair (1935, *a*) modified as follows:

Saponification of the acetone-insoluble fraction was carried out in a centrifuge tube and the mixed fatty acids extracted with petroleum ether until the water solution was clear, the petroleum ether being drawn off each time directly into a 50 cc. volumetric flask.   Two 3 cc. aliquots were taken for determination of amount and iodine number, respectively.   The remaining 46 cc. of the petroleum ether solution were evaporated to about 3 cc., transferred quantitatively to a 15 cc. centrifuge tube, and concentrated

to 1 cc.; 7 cc. of redistilled 95 per cent alcohol were added and the volume reduced to 7 cc.; 0.7 mg. of solid lead acetate was added for each mg. of fatty acid, the solution boiled until the volume was reduced to 6 cc., and the material insoluble in hot alcohol centrifuged off and washed as described. • The lead soaps of the solid fatty acids were allowed to precipitate from a volume of alcohol equal to 0.1 cc. per 10 mg. of fatty acids by keeping them at a temperature of 15–17° for at least 4 hours. 2 hours were allowed for the second precipitation. The rest of the procedure was carried out exactly as described.

Determination of Elaidic Acid—Elaidic acid is an unsaturated acid with an iodine number of 90 but, unlike most unsaturated acids, it is a solid; its lead salt is insoluble in cold alcohol and separates along with the lead salts of the saturated acids from a mixture of naturally occurring fatty acids. The percentage of elaidic acid in the solid acids is calculated by dividing the iodine number of the solid fraction by 90, the iodine number of the elaidic acid; multiplication of this value by the percentage of solid acids gives the percentage of elaidic acid in the total phospholipid fatty acids.

Results

Apparent Solid Unsaturated Fatty Acid Content of Tumor Phospholipids of Rats on Calf Meal Diet—Since an absolute separation of solid and liquid fatty acids is not obtained by the micromethod employed, the apparent percentage of solid unsaturated acids of tumors from rats on the stock diet of calf meal must be determined. Table I shows the percentage of solid acids in the phospholipid fatty acids (corrected for the amount of alcohol-insoluble material present), the iodine number of the solid acids, and the calculated percentage of solid unsaturated acids in the phospholipid fatty acids from nine tumors 27 to 49 days old. The average percentage of solid acids in these control tumors is 31.3 ± 2.2; the apparent solid unsaturated acid content of the phospholipid fatty acids averaged 4.0 ± 0.8.

Elaidic Acid Content of Tumor Phospholipids from Rats Placed on Elaidin at Inoculation—As Sinclair (1935, b) has pointed out, if phospholipids function as intermediates in the metabolism of fat, three criteria must be satisfied. In the first place, it must be shown that food fatty acids enter the tissue phospholipids; in the

TABLE I

Apparent Solid Unsaturated Fatty Acid Content of Tumor Phospholipids of Rats Fed on Calf Meal Diet

Animal No.	Tumor age	Solid acids	Iodine No.	Solid unsatu-rated acids
	days	*per cent*		*per cent*
187-NM	27	30.3	12.5	4.2
173-BV	28	30.1	11.9	4.0
197-2-RV	28	31.4	12.8	4.5
196-2-RV	31	31.3	13.9	4.8
200-LV	31	31.8	14.8	5.2
192-NM	31	34.1	10.7	4.1
172-2-RV	32	31.9	7.9	2.8
197-NM	38	32.4	12.2	4.4
197-2-LV	49	28.5	8.0	2.5
Average....................		31.3	11.6	4.0
Standard deviation...........		±2.2	±2.4	±0.8

TABLE II

Elaidic Acid Content of Tumor Phospholipids from Rats Placed on Elaidin at Inoculation

Animal No.	Tumor age	Solid acids	Iodine No.	Elaidic acid
	days	*per cent*		*per cent*
194-LV	28	37.2	50.9	21.1
195-LV	28	32.8	52.5	19.1
193-2-RV	28	38.6	46.5	20.0
183-RV	29	30.4	47.5	16.3
184-BV	29	35.7	45.8	18.2
185-LV	29	35.3	49.9	19.6
193-LV	33	26.7	45.3	13.5
194-LV-2-RV	35	35.9	46.6	18.6
193-NM	35	28.1	45.0	14.1
193-RV	35	33.0	50.0	18.3
193-BV	40	34.4	46.5	17.8
194-NM	68	37.0	43.6	17.9
Average....................		33.7	47.5	17.9
Standard deviation...........		±3.6	±2.6	±2.2

second place, that the rate of entrance of fatty acids into the phospholipids is rapid; in the third place, that the rate of disappearance of the fatty acids from the phospholipids is also rapid. In order to show that elaidic acid enters the tumor phospholipids, rats were taken at the time of inoculation with the tumor and placed on Diet 290-C (Sinclair, 1935, *b*) which consisted of casein 18 per cent, yeast 8 per cent, 1:1 cod liver oil and corn oil 4 per cent, and elaidin 70 per cent by calories. 1 gm. of salt mixture (Sinclair, 1931) was added for each 100 calories. From 29 to 68

TABLE III

Elaidic Acid Content of Tumor Phospholipids from Rats Raised on Elaidin

Animal No.	Tumor age	Solid acids	Iodine No.	Elaidic acid
	days	*per cent*		*per cent*
235-L-2-V	31	38.8	47.8	20.7
237-NM	31	35.6	45.6	18.0
237-RV	31	35.2	51.4	20.1
235-LV	31	36.5	49.3	20.0
237-L-2-V	32	29.4	47.7	15.6
235-NM	32	31.6	52.2	18.3
236-BV	32	34.3	52.2	19.9
237-R-2-V	36	33.6	44.8	16.7
237-LV	36	28.8	45.7	14.6
236-R-2-V	36	33.7	49.7	18.6
236-LV	45	26.0*	64.1*	18.5
235-R-2-V	45	31.7	49.8	17.6
Average		32.9	50.2	18.2
Standard deviation		±1.8	±4.9	±1.8

* These values are excluded on the basis of the standard deviation.

days later, depending on the growth rate of the tumors, they were removed and analyzed. The results are given in Table II. The average percentage of solid acids in these tumors from animals on the elaidin diet is 33.7 ± 3.6; the percentage of elaidic acid in the phospholipid fatty acids averaged 17.9 ± 2.2. Thus elaidic acid has entered the phospholipid fatty acids of the tumor and the first criterion is satisfied.

Elaidic Acid Content of Tumor Phospholipids from Rats Raised on Elaidin—Since the value of 17.9 ± 2.2 per cent for the elaidic acid content of the phospholipid fatty acids of tumors from animals

placed on the elaidin diet at the time of inoculation is low com-
pared with values found by Sinclair (1935, b) for muscle, it seemed
of interest to determine the elaidic acid content of tumors from
animals which were born and raised on Diet 290-C.   Female rats
were placed on the diet at mating and the males of the resultant
litters were kept on the diet through inoculation and growth of
the tumors.   The results are shown in Table III.   The average
percentage of solid acids in these tumors from animals born on
the elaidin diet is 32.9 ± 1.8; the percentage of elaidic acid in the

TABLE IV

Elaidic Acid Content of Tumor Phospholipids from Rats on Calf Meal Diet at Inoculation and Later Transferred to Elaidin

Animal No.	Tumor age	Time on elaidin	Solid acids	Iodine No.	Elaidic acid
	days	*days*	*per cent*		*per cent*
181-BV	31	2	34.2	32.8	12.5
171-LV	32	2	29.8	14.4	4.8
172-LV	32	2	30.5	15.2	5.2
171-2-RV	32	2	32.2	28.6	10.2
171-NM	32	2	28.8	19.4	6.2
172-NM	32	2	28.2	14.9	4.7
174-2-LV	33	5	35.7	41.9	16.6
181-LV	34	5	28.2	39.4	12.3
181-NM	34	5	32.6	34.5	12.5
188-NM	37	8	29.2	26.7	8.7
188-LV	37	8	30.0	35.5	11.8
Average................................			30.9		
Standard deviation....................			±2.4		

phospholipid fatty acids averaged 18.2 ± 1.8, a value which is not
significantly higher than that for tumors from animals placed on
the diet at inoculation.

Rate of Entrance of Elaidic Acid into Tumor Phospholipids—The
rate of entrance of elaidic acid into the tumor phospholipids was
determined by transferring tumor animals from the stock calf
meal diet to elaidin and removing the tumors in 2, 5, and 8 days.
The results are shown in Table IV.   It is evident from the data in
Table IV that the entrance of elaidic acid into the phospholipid
fatty acids of tumor tissue is a relatively slow process, since it is
incomplete even after 8 days.

Rate of Replacement of Elaidic Acid from Tumor Phospholipids by Saturated Fatty Acids—Rats were placed on the elaidin diet on the day of tumor inoculation.   29 days later they were transferred to Diet 290 (Sinclair, 1935, b) containing 51.2 per cent beef tallow, and the tumors removed in 3, 4, and 7 days.   The results are shown in Table V.   Replacement of elaidic acid by saturated fatty acids is incomplete even after 7 days, since the percentage of elaidic acid is above 4.0 (the control value for the percentage of solid unsaturated acids).

TABLE V

Elaidic Acid Content of Tumor Phospholipids from Rats Placed on Elaidin Diet at Inoculation and Later Transferred to Beef Tallow

Animal No.	Tumor age	Time on beef tallow	Solid acids	Iodine No.	Elaidic acid
	days	*days*	*per cent*		*per cent*
195-2-RV	32	3	30.7	27.2	9.3
183-2-RV	33	4	31.4	27.0	9.4
184-2-LV	33	4	30.3	23.3	7.9
183-LV	33	4	28.8	16.1	5.2
185-2-RV	36	7	24.8	20.0	5.5
184-NM	36	7	26.4	15.8	4.6
185-BV	36	7	32.0	16.3	5.8
183-BV	36	7	29.9	16.1	5.4
Average................................			29.3		
Standard deviation.........			±2.3		

DISCUSSION

When rats are raised on a diet containing 70 per cent elaidin, their tumor phospholipids contain as much as 18.2 per cent elaidic acid (Table III).   This value is low compared with those found by Sinclair (1935, b) for liver (average 26.2 per cent) and muscle (average 29.8 per cent) of rats on the same diet.   There are several possible explanations for this lower value.   In the first place, each tissue may possess the ability to select those fatty acids (or phospholipids containing those fatty acids) which are adapted to its own particular needs.   For example, no elaidic acid could be detected in the phospholipids of the red blood cells of cats fed elaidin, although the phospholipid fatty acids of the blood plasma contained as much as 37 per cent elaidic acid (Sinclair, 1936).

Likewise, brains of rats on the elaidin diet contain much less elaidic acid than do other organs (McConnell and Sinclair, 1937). The possibility that elaidic acid may enter only the lecithin fraction of the phospholipids is being investigated.   Also, it is possible that elaidic acid may enter the tumor phospholipids and then undergo partial saturation owing to conditions within the tumor, since the presence in tumors of reducing substances such as glutathione and ascorbic acid has been conclusively demonstrated (Kellie and Zilva, 1936; Boyland, 1936).   However, this should cause a higher percentage of solid acids in the elaidin-fed animals, whereas the average values for solid acids in Tables II and III are not significantly higher than the control value in Table I.

The rate of entrance of elaidic acid into tumor phospholipids is slow compared with that in liver, in which the process is virtually complete within 2 days (Sinclair, 1935, b).   However, the rate of entrance is faster than it is in muscle, since the tumor phospholipids of one animal at 2 days and of others at 5 days contain more elaidic acid calculated as per cent of the maximum (18.2 per cent) than do the muscle phospholipids at 6 and 7 days (Sinclair, 1935, b).   Moreover, the rate of disappearance of elaidic acid from the tumor phospholipids, while slower than that for liver, is faster than that for muscle, being practically complete after only 7 days, whereas Sinclair (1935, b) found it still incomplete in about the same length of time.

 · When we decided to feed elaidin to tumor-bearing rats, we hoped that it would lead to as decisive results concerning the nature of the phospholipids of tumor as it had to those of liver and muscle.   Unfortunately, such clear cut results have not been obtained with tumor.   An explanation of the somewhat faster rate of turnover of elaidic acid in tumor phospholipids as compared with muscle phospholipids may lie in the fact that the maximum content of elaidic acid in tumor tissue is lower than it is in muscle. However, since the rate is slow compared with that in liver, one can conclude that the phospholipids of tumor are mainly of the non-metabolic rather than of the metabolic type.   Such phospholipid is concerned with the essential cellular make-up rather than with burning of the fatty acids for energy.

Dickens and Šimer (1930, 1931), in studying the respiratory quotients of various tissues, made the generalization that all

normal tissues which showed high glycolytic activity anaerobically, respired in the presence of oxygen at a respiratory quotient of 1. Therefore, the main respiratory activity of such tissues appeared to consist in carbohydrate combustion or oxidation of the lactic acid or some precursor, while under anaerobic conditions lactic acid accumulated.   Tumor tissue, in spite of having a very high glycolytic activity, respired at a moderately low respiratory quotient, and this was taken as evidence that a failure to oxidize carbohydrate (or lactate) was characteristic of tumor tissue. Respiratory quotients as low as 0.77 to 0.78 were found by Dickens and Šimer (1930, 1931). . Moreover, Dickens and Greville (1933) showed that the respiratory quotient of respiring Jensen rat sarcoma could not be due to protein metabolism, since the ammonia and urea production were very small.   At this time it seemed possible that the low respiratory quotients of tumor tissue might be due in part to fat metabolism, since the phospholipid content of tumors is high and phospholipids have been assumed to take part in the metabolism of fats.

More recently, however, Jares (1935), using the technique of Dickens and Šimer (1930, 1931), has reported values for respiratory quotients of tumor tissue considerably higher than those found by these workers; namely, average values of from 0.83 to 0.95 for various mouse carcinoma and rat and mouse sarcoma. For rat Carcinosarcoma 256, the tumor used in our experiments, he found an average value of 0.89.   The finding of these high respiratory quotients would exclude the possibility of appreciable fat metabolism occurring within the tumor, which is in agreement with our conclusion that tumor phospholipids are mainly of the non-metabolic type.   One can only speculate concerning the function of this type of phospholipid but presumably it is concerned with the physicochemical properties of the cell.

The ratio of the solid (saturated) to the liquid (unsaturated) fatty acids of the phospholipids of the rat was found by Sinclair (1935, a) to be constant, at least for liver, skeletal muscle, and kidney.   When the degree of unsaturation of the phospholipids in tumors was found to be much lower than that of the phospholipids in the muscles of the host (Haven, 1935), it seemed possible that this ratio might have a value different from that of approximately 30 per cent solid acids to 70 per cent liquid acids

found by Sinclair. However, as the data in Tables I to V show, this ratio holds for tumor tissue as well as muscle, since the average of all values for liquid acids (determined, but not given in the tables) was approximately 70 per cent, the recoveries of both solid and liquid acids being from 95 to 99 per cent. Thus the lower degree of unsaturation of the phospholipid fatty acids of tumor tissue is due to the presence of acids which are less unsaturated rather than to a larger amount of saturated acids.

SUMMARY

1. Feeding elaidin to rats, the method used by Sinclair in classifying phospholipids into the metabolic type which predominates in the liver and the non-metabolic type which predominates in muscle, indicates the following conclusions about tumor phospholipids: (a) Elaidic acid enters the tumors to the extent of about one-fifth of the phospholipid fatty acids. (b) The rate of entrance of elaidic acid into and disappearance from the tumor phospholipids is slow compared with that for liver phospholipids. (c) The phospholipids of tumor are mainly of the non-metabolic type, having to do with cellular structure rather than with burning of fatty acids for energy.

2. The ratio of the solid (saturated) to the liquid (unsaturated) fatty acids of rat tumor phospholipid is the same as that of rat muscle phospholipid, namely 30:70.

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PASSAGE OF ELAIDIC ACID THROUGH THE PLACENTA AND ALSO INTO THE MILK OF THE RAT

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Up to the present, the proof that the placenta of the dog (1), the rabbit (2), and the rat (3, 4) is permeable to the fatty acids in the maternal circulation has rested on the demonstration that the degree of unsaturation of the fatty acids in the fetus is influenced by the nature of the fat fed to the mother. While that evidence would seem to be sufficient and has generally been so regarded, nevertheless it seemed to us worth while to provide further proof by showing that the readily distinguishable fatty acid, elaidic acid, is present in the new born young of pregnant rats to which it had been fed. In the same way, the determination of the elaidic acid content of young rats suckled by lactating females which had been fed elaidin offered a means of further demonstrating that ingested fatty acids pass through the mammary gland into the milk. For recent direct evidence of the effect of food fat on the composition of milk fat, consult the papers by Hilditch (5) and Ellis (6) and their coworkers.

Two groups of experiments were carried out. In one, the elaidic acid content of the liver phospholipids in new born rats and after a suckling period of about 3 weeks was determined; in the other, the total elaidic acid content of young rats at birth and at 10 days of age was determined.

EXPERIMENTAL

The adult female rats were fed on Diet 290-C, elaidin, consisting of casein 28.1 per cent, salt mixture (7) 6.3 per cent, yeast 14.4 per cent, elaidin (8) 48.4 per cent, corn oil 1.4 per cent, and cod liver oil 1.4 per cent. On this diet, 70 per cent of the calories were

derived from elaidin.   The corn oil and cod liver oil were fed to supply adequate amounts of unsaturated fatty acids and of the fat-soluble vitamins.   This diet was not quite as good as the stock diet of G. L. F. calf meal (9) for fetal growth, since the average birth weight of thirty-two young born of mothers on the elaidin diet was 4.70 gm. as against 5.11 gm. for the stock diet.   Similarly, the average weight when 3 weeks old was slightly less for young suckled by elaidin-fed mothers than for stock animals.   Nevertheless, the diet kept the females in good condition and enabled them to have as many as four successive litters.

To prevent the new born rats from suckling, the pregnant females were kept in a special cage for the last day or so prior to delivery, the cage being so constructed that the young were segregated from the mother immediately after birth.

When determining the elaidic acid content of the liver phospholipids, the following procedure was used: The livers (from about twenty new born rats and from two rats 3 weeks old) were ground thoroughly with sand and transferred to a suitable flask with 95 per cent alcohol.   The lipids were extracted by boiling with three lots of alcohol.   The combined filtrates were concentrated, transferred to a 50 cc. centrifuge tube, and evaporated almost to dryness.[1]   Precautions were taken to prevent overheating and exposure to air.   Acetone (25 cc.) was added and then 0.3 cc. of 20 per cent $MgCl_2$ in 95 per cent alcohol.   The precipitated phospholipids were stirred up well in the acetone and then centrifuged.   The phospholipids were reprecipitated from petroleum ether with acetone and centrifuged.   The precipitate was stirred up in a little petroleum ether and then 25 cc. of alcohol and 0.2 cc. of saturated aqueous KOH solution were added.   The centrifuge tube was covered with a small funnel and heated on the steam bath for at least $\frac{1}{2}$ hour.   When the volume had become reduced to about 4 cc., 2 cc. of distilled water were added and the

[1] The procedure outlined was the final one chosen after trying out several modifications of various steps.   At first the alcohol extract was evaporated just to dryness in an Erlenmeyer flask and the lipids extracted with petroleum ether.   While the resultant removal of alcohol-soluble non-lipid material is essential if phospholipid is to be oxidized directly, it is not necessary if the phospholipid is to be saponified.

heating continued for a few minutes, a gentle stream of nitrogen being passed through the tube to prevent oxidation. Sulfuric acid (0.6 cc. of 25 per cent by volume) was added. The liberated fatty acids together with the unsaponified lipids were extracted by boiling gently with a mixture of chloroform and petroleum ether and with petroleum ether alone. The combined solvents, aspirated into another 50 cc. centrifuge tube, were evaporated to about 1 cc. Acetone (30 cc.) and 3 drops of 4 per cent $MgCl_2$ in alcohol were added. The flocculent precipitate of the unsaponified acetone-insoluble lipids[2] was centrifuged down and the clear acetone solution of the phospholipid fatty acids[3] was aspirated into a 50 cc. volumetric flask. After removal of aliquots for oxidation and for iodine number determination, the remainder was separated into solid and liquid acids by the procedure already described (10). The percentage of elaidic acid was calculated from the percentage[4] and iodine number of the solid acids (8).

To determine the elaidic acid content of the total fatty acids, an aliquot of the combined alcohol and ether extracts of the finely ground body of the rat was saponified as above. After extraction of the unsaponifiable material with petroleum ether, the liberated fatty acids were extracted and made up to a volume of 50 cc. in petroleum ether. Aliquots were removed for oxidation and iodine number determinations and the remainder was separated into alcohol-soluble and alcohol-insoluble lead salts as

[2] As pointed out in an earlier paper (10), part of the lipids precipitated by acetone are not saponified by the customary methods of saponification. The nature of these unsaponifiable acetone-insoluble lipids is not known but, on the basis of their known resistance to saponification (11), it is probable that sphingomyelins and cerebrosides make up a part at least. If not removed by acetone, the unsaponified lipids separate out as a flocculent precipitate from the hot alcoholic solution of the lead salts of the fatty acids. The acetone treatment removes most, but not all, of the unsaponified material.

[3] For convenience, the term "phospholipid fatty acids" is used to designate the fatty acids split off from the saponified acetone-insoluble material. Strictly speaking, these fatty acids have been split off from the lecithins and cephalins.

[4] The fatty acid equivalent of the material insoluble in hot alcohol is subtracted from the previously determined amount of mixed fatty acids before the percentage of solid acids (10) is calculated.

described above.   Instead of recrystallizing the alcohol-insoluble soaps from alcohol, the fatty acids were regenerated from the lead soaps, extracted with ether, transferred to a clean 15 cc. centrifuge tube, and taken up in alcohol after removal of the ether.   Lead acetate was again added and the lead soaps allowed to crystallize

TABLE I

Elaidic Acid Content of Liver Phospholipid Fatty Acids

Group No.	Phospholipid fatty acids			
	Amount in moist liver	Solid acids (corrected)	Iodine No. of solid acids	Elaidic acid
	per cent	*per cent*		*per cent*
I. Controls; new born of fe-	1.01	35	19	8
males fed on stock calf meal	1.52	40	7	3
diet	1.26	37	15	6
	1.19	36	16	6
Adults; average taken from previous paper (8)		37	7	3
II. New born of females fed on	1.01	43	37	17
Diet 290-C, elaidin	0.66	39	43	19
	1.02	36	24	10
	0.99	38	35	15
	1.10	39	43	19
	1.06	39	43	19
	1.06	36	32	13
III. 3 wk.-old rats born of and	2.32	43	58	28
suckled by mothers fed on	2.22	47	60	31
Diet 290-C, elaidin	2.13	42	47	22
	2.25	48	54	28
	2.11	44	49	24
IV. 3 mo.-old rats reared on	1.69	50	53	29
Diet 290-C, elaidin	2.64	45	55	28
	2.38	44	54	26
	2.31	45	50	25

out in the cold.   It was hoped that formation anew of the lead soaps instead of a simple recrystallization would diminish the contamination of the saturated acids by unsaturated acids in the control experiments.   The iodine numbers of the solid acids in Table II are in fact somewhat lower than those in Table I but the difference is not as great as had been hoped for.

Results and Comments

The data concerning the phospholipid fatty acids of the liver and those concerning the total fatty acids of the entire animals are given in Tables I and II, respectively.

Inspection of the last column of each of these tables will show that the percentage of elaidic acid in both the phospholipid fatty acids of the livers and the total fatty acids of the entire animals is significantly greater in the new born rats and several times greater

TABLE II

Elaidic Acid Content of Total Fatty Acids in Rat

Group No.	Total fatty acids			
	Amount	Solid acids (corrected)	Iodine No. of solid acids	Elaidic acid
	per cent	*per cent*		*per cent*
I. New born of females on calf meal diet	0.75	37	10	4
	0.89	35	13	5
	0.68	36	11	4
	0.66	37	8	3
II. New born of females on Diet 290-C, elaidin	0.79	37	25	10
	0.74	38	29	12
	0.78	38	22	9
	0.74	36	26	10
III. 10 day-old young of females on calf meal diet	4.2	30	9	3
	3.4	34	7	3
	4.5	33	8	3
	4.1	33	7	3
IV. 10 day-old young of females on Diet 290-C, elaidin	9.9	71	78	61
	9.6	72	78	63
	8.6	74	72	59
	9.6	70	79	61

in the suckling rats than the apparent elaidic acid content[5] of the controls. One may therefore safely conclude that elaidic acid

[5] Owing to the fact that the alcohol-insoluble lead salts of the saturated fatty acids (solid acids) are always contaminated with some unsaturated acids, presumably mostly oleic acid, the mixed fatty acids from the tissues of animals on ordinary diets appear to contain a small amount of elaidic acid. There is no reason to believe at present that elaidic acid is actually present in such animals.

passes from the maternal circulation through the placenta into the fetal circulation and through the mammary gland into the milk. Since this unnatural fatty acid passes through the placenta and mammary gland of the rat, it would seem likely that all the natural fatty acids occurring in the maternal blood do so also.

It will be seen from Table I that the percentage amount of phospholipid fatty acids in the liver approximately doubles during the first 3 weeks of postnatal life. Part, but not all, of this increase is undoubtedly due to the decrease in the water content of the liver (12). During the same period, there is an increase in the percentage of elaidic acid in the fatty acids of the liver phospholipids (from an average of 16 to 27 per cent). Consequently, the total amount of elaidic acid combined as phospholipid in the liver increases about 27-fold during the first 3 weeks after birth, although the total amount of liver tissue increases only about 6-fold. The explanation of this great increase in the amount of phospholipid in the liver, especially in that containing elaidic acid, is by no means clear. It is conceivable that it is due to the greater amount of fat, and therefore of elaidic acid, being burned. On the other hand, the shift in the elaidic acid content of the liver phospholipids after birth may be evidence of a selective permeability of the placenta, or of selection in the synthesis of phospholipid, in favor of the natural fatty acids.

It is noteworthy that while the phospholipids contain practically all of the fatty acids in the body of the new born rat (3), they contain only about 10 per cent of the total fatty acids present in the 10 day-old rats, the remaining 90 per cent being neutral fat.

SUMMARY

When a diet rich in elaidin was fed to mother rats, elaidic acid was found to make up 16 per cent of the phospholipid fatty acids of the liver and 11 per cent of the total fatty acids of the entire bodies of new born young. If the young are allowed to suckle, after 10 days elaidic acid makes up about 61 per cent of the total fatty acids of the animal; after 3 weeks the elaidic acid content of the liver phospholipid fatty acids amounts to 27 per cent. It is concluded that elaidic acid passes through the placenta and into the milk of the white rat.

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EVIDENCE OF SELECTION IN THE BUILDING UP OF BRAIN LECITHINS AND CEPHALINS

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Several years ago it was shown that, of the various organs studied, the brain was the only one which did not show a clear cut relationship between the composition of the phospholipids and the nature of the food fat (1). The brain phospholipids appeared to exhibit the constancy of composition which was thought by Terroine and coworkers (2, 3) to hold for all tissue phospholipids. Meanwhile it has been found that elaidic acid can be used with success as a means, not only of demonstrating that ingested fatty acids become incorporated into the tissue phospholipids, but of measuring the rate of phospholipid turnover (4, 5). Obviously it was of considerable interest to apply the elaidic acid procedure to the phospholipids, or more precisely, the lecithins and cephalins of the brain.

A few preliminary experiments indicated that there was little, if any, elaidic acid in the lecithins and cephalins of the brains of rats which had been raised from weaning age on a diet rich in elaidin. In view of the fact that a large part of the brain development takes place during the first 3 weeks of postnatal life (6), it was decided to supply large amounts of elaidic acid throughout the entire period of development. This was accomplished by feeding an adequate diet rich in elaidin to adult female rats during the gestation period and while the young were suckling. As shown by the preceding paper (7), elaidic acid passes through the placenta and also into the milk. Groups of rats were taken at birth, when about 3 weeks old, and when about 3 months old.

EXPERIMENTAL

The diet used for the mother rats and for rearing the young is described in the preceding paper (7).

The rats were killed by decapitation.   The brains were ground with sand and transferred to a suitable flask with 95 per cent alcohol.   The total lipids were extracted by boiling with three lots of alcohol and two of chloroform.   The combined solvents were evaporated, the lipids fractionated into acetone-soluble and acetone-insoluble fractions, the latter saponified, the unsaponified lipids[1] separated from the lecithin and cephalin fatty acids, and the elaidic acid contents of the latter determined according to the directions given elsewhere (4, 5, 7).

Results

The data showing the percentage amount of lecithin and cephalin fatty acids, the percentage and iodine number of the solid acids, and the calculated percentage of elaidic acid are given in Table I.

There is little need to comment on the pronounced increase in the lecithin and cephalin fatty acid content of the brain during the first 3 weeks of life and the further increase between weaning and maturity, except to point out that these data agree quite well with comparable ones calculated from the extensive data of Koch and Koch (8).

The average value of the percentage (corrected) of solid acids in the lecithin and cephalin fatty acids from the brains of all of the rats analyzed amounts to 40.6 ± 3.5.   Although most of the values in the adults (Group IV) are lower than in the new born rats (Groups I and II), the data do not permit one to conclude whether or not the difference is significant.

With respect to the iodine numbers of the solid acids, in only three of the seven experiments on rats at birth, and in only three out of five of the 3 week-old rats, are the values higher in the rats getting elaidic acid than the highest value found in the controls. Such differences as do occur are so small that it would obviously

[1] It is thought probable that, in the case of the brain at any rate, most of the acetone-insoluble lipids which are not saponified by the procedure employed (7) consist of sphingomyelins and cerebrosides.   The fact that the amount of acetone-insoluble material in the fatty acids increased several fold between birth and adult age is in harmony with that belief, since myelin formation in the brain of the rat takes place after birth (6).   This problem is being investigated further.

take a large number of experiments to prove whether or not they are statistically significant. Since the iodine numbers are in-

TABLE I

Elaidic Acid Content of Lecithin and Cephalin Fatty Acids in Brain

Group No.	Lecithin and cephalin fatty acids			
	Amount in moist brain	Solid acids (corrected)	Iodine No. of solid acids	Elaidic acid
	per cent	per cent		per cent
I. Controls; new born young of females fed on stock calf meal diet	1.20	43	14	7
	1.21	35	7	3
	1.29	37	4	2
	0.93	45	5	3
	1.23	44	11	5
	1.32	42	12	6
II. New born young of females fed on Diet 290-C, elaidin	1.00	45	13	7
	0.95	43	11	5
	1.09 .	45	16	8
	1.19	45	15	8
	1.23	44	12	6
	1.21	45	15	8
	1.32	42	14	7
III. 3 wk.-old rats born of and suckled by females fed on Diet 290-C, elaidin	2.76	35	11	4
	2.33	40	15	7
	2.36	45	11	6
	1.48	36	19	8
	2.66	39	19	8
IV. 3 mo.-old rats reared on Diet 290-C, elaidin	2.87	37	30	12
	3.20	40	27	12
	3.04	35	22	9
	3.00	37	23	10
	2.85	40	21	9
	3.22	(41)*	17	(8)*
	3.10	44	16	8
	2.38	37	22	9
	3.33	38	18	8

* In this experiment the percentage of solid acids was calculated by subtracting the percentage of liquid acids from 100.

volved in the calculation of the elaidic acid percentages, it is not possible from these data to say definitely that elaidic acid has been used in the building up of lecithins and cephalins in the brains of

rats during the period of intrauterine development and during the first 3 weeks of postnatal life.   It is thought likely that such is the case, to some extent at least.

In the brains of the nine mature rats, on the other hand, the solid acids from the lecithins and cephalins all had iodine numbers which were above the range for the controls and most were suffi-
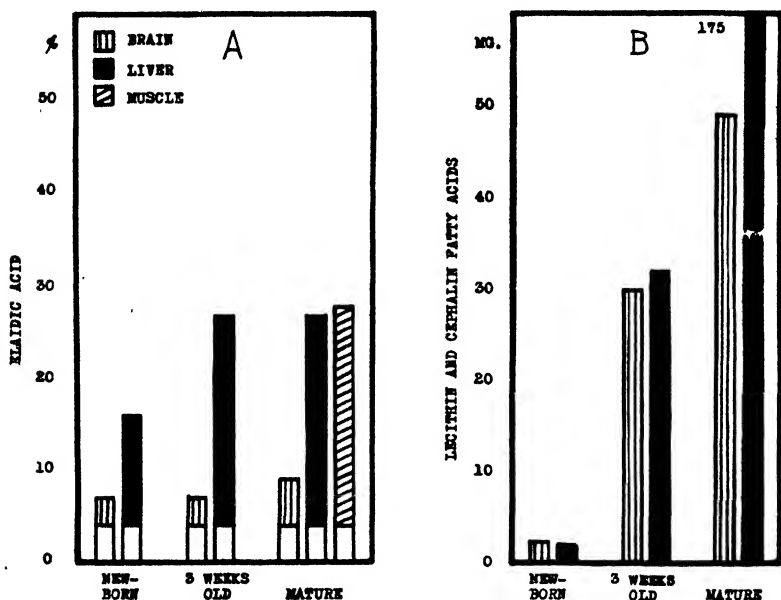
FIG. 1. A, the percentage of elaidic acid in the fatty acids of the lecithins and cephalins from the brains, livers, and muscles of rats fed large amounts of elaidin throughout their entire prenatal and postnatal life. The un- shaded portions show the average apparent elaidic acid content of the tissues of control animals. B, the average total amount of lecithin and cephalin fatty acids in the brain and liver of a rat at birth, at 3 weeks of age, and at maturity.

ciently high to remove all reasonable doubt as to the presence of elaidic acid.

However, the amount of elaidic acid in the lecithins and cepha- lins of the brain, even in rats which have been supplied with elaidic acid throughout the entire period of growth from the fertilized egg to maturity, is much less than is present in the lecithins and cephalins of the liver and muscle of the same animals.   This fact is

unmistakably demonstrated by Fig. 1, *A* which shows the average percentage of elaidic acid in the phospholipids of the brain, liver, and muscle of the same, or comparable, groups of animals.   The data for liver and muscle have been taken from previous papers (5, 7).   Fig. 1, *B* shows the approximate amount of lecithin and cephalin fatty acids in the brain and liver of a rat at birth, at 3 weeks of age, and at maturity.   On comparing the two sections of Fig. 1, it will be seen that after the first 3 weeks of postnatal life, during which time the absolute increase in lecithins and cephalins was about the same in brain and liver, elaidic acid makes up about 27 per cent of the phospholipid fatty acids in the liver but only about 7 per cent of those in the brain.

Even in the liver and muscle, however, the percentage of elaidic acid in the total phospholipid fatty acids is by no means the same ·as that of the ingested fat, which amounts to about 80 per cent. To what extent the circulating lipids of the rat, which are the immediate source of supply for the organs, differ from the ingested fat in their elaidic acid content is not at present known.   If one may judge from the findings on the cat (5), the blood of the rat on the elaidin-rich diet probably contains both phospholipids and fat richer in elaidic acid than are the phospholipids of the brain, liver, and muscle.   Not less than two-thirds of the fatty acids in the depot fat consists of elaidic acid.   It would seem likely, therefore, that there has been a considerable selection of fatty acids from those available in the circulating blood in the building up of tissue phospholipids.   It has already been pointed out (4) that, among other facts, the constancy of the ratio of solid to liquid acids in the phospholipids and the preference for the highly unsaturated acids are suggestive of such a selection.

The present results indicate that the selection of fatty acids (or of phospholipids themselves) in the building up of the constituent phospholipids of the tissues is much more rigorous in the brain than in the liver and muscles.   The difference between brain and muscle is perhaps more significant than that between brain and liver, since the liver contains a considerable amount of metabolic phospholipid (in the sense of acting as a fuel) and, furthermore, may take up elaidic acid from the portal circulation.

Nothing is known as to the factors involved in the selective synthesis of tissue phospholipids.

Since elaidic acid makes up approximately 80 per cent of the fatty acids ingested and burned by the rats, the fact that only comparatively small amounts are present in the brain phospholipids would seem to indicate quite clearly that the function of most of the lecithins and cephalins in the brain, whatever it may be, is not that of an intermediary in fatty acid catabolism.

SUMMARY

In rats provided with large amounts of elaidic acid throughout the entire period of prenatal and postnatal development, the elaidic acid content of the fatty acids in the lecithins and cephalins of the brain was found to be only about one-fourth of that of the liver and muscles. Thus it appears that there is a greater degree of selection in the building up of brain phospholipids than those of liver and muscle.

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COLORIMETRIC ESTIMATION OF GUANIDINE-LIKE SUBSTANCES IN THE URINE

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Experimental work on the presence of guanidine bases in the urine had its beginning in 1906, when Achelis (1) claimed to have demonstrated the presence of methylguanidine in the urine of men, dogs, and horses. Other workers continued the work along this line, using similar methods, but the work of Baumann and Ingvaldsen (2), in 1918, showed conclusively that the methods used by these investigators converted creatinine and creatine into guanidines, and made it seem very doubtful whether the results obtained by the previous investigators were of any value.

In 1920, Findlay and Sharpe (3) and also Sharpe (4) described picric acid precipitation methods, by which they, along with other workers, claimed to have demonstrated guanidine bases in the urine of normal individuals, as well as variations in the guanidine excretion in certain diseases. However, the work of Greenwald (5) and also White (6) showed quite conclusively that the picric acid precipitation method is of little value for the determination of guanidine and its methyl derivatives, in the presence of creatinine. Finally, Kuen (7), in 1927, using a method whereby the guanidine is precipitated with picrolonic acid, failed to show the presence of guanidine bases in urine. He admitted, however, that his method is not sensitive for quantities of guanidine under 50 mg. per liter.

Weber (8), in 1928, described a new method for the colorimetric estimation of guanidine bases in urine based on the principle of his own blood method. His method consists essentially in removing certain salts and part of the color of the urine by treatment with lead acetate, removing the lead acetate with sodium phosphate,

extracting the guanidine with norit in a basic solution, and colorimetrically estimating the guanidine bases with Weber's ferricyanide reagent (9). He observed that both norit and blood charcoal slightly convert creatinine into guanidine bases, the charcoal having the greater action. He stated that under the conditions prescribed by his method, the amount of creatinine so converted into guanidine is proportional to the concentration of creatinine, and amounts to about 0.70 mg. of guanidine (determined as guanidine) per 100 mg. of creatinine. His method gave 85 per cent recovery of added guanidine.

Using this method, Weber found that the guanidine excretion of normal males amounts to between 10 and 20 mg. per 24 hours, and varied greatly even with each individual. He furthermore stated that he had no definite proof that the substance determined by his method is guanidine or any of its derivatives.

In 1931, Stockholm and Cercedo (10) gave a preliminary report on a new precipitation method for the determination of guanidine bases in urine. They recovered from 66 to 80 per cent of the guanidine bases added (depending on the guanidine derivative used), but it is significant to note that in their recovery experiments they added from 200 to 800 times the amount of guanidine normally found in human urine. They gave no values for human urine.

EXPERIMENTAL

Use of Acid Alcohol to Release the Guanidines Adsorbed on the Norit—While in most respects the method outlined by Weber (8) is quite satisfactory, in some urines the final extract is always brown in color, the color being sufficient to give errors as high as 100 per cent. Experiments were carried out whereby the guanidines, adsorbed on the norit, were released by means of boiling acid alcohol, instead of cold alcohol. Introducing this change into Weber's method, recoveries of methylguanidine added to normal urine amounted to about 85 per cent (see Table I). A comparison of the recovery with boiling alcohol, and with four 10 cc. portions of cold alcohol (as described by Weber), showed that the hot alcohol gave about 2 per cent greater recovery. Furthermore, and most important, the final product, when the hot alcohol was used, was colorless (or but slightly colored) with all the urines

analyzed.   The slight color, obtained with a few urines, can usually be eliminated by careful evaporation of the final extract.

Conversion of Creatinine into Guanidines by Norit—Weber found that the amount of guanidines formed from creatinine (by norit in a basic solution) was proportional to the creatinine concentration, and that this conversion amounts to about 0.70 mg. per

TABLE I

Recovery of Methylguanidine Added to Urine (Released with Boiling Alcohol)

Methylguanidine		Recovered
Added per 100 cc. of urine	Recovered per 100 cc. of urine	
mg.	*mg.*	*per cent*
1.50	1.29	86
1.50	1.31	87
1.50	1.27	85
1.50	1.33	88
1.50	1.25	84
1.50	1.34	89
3.33	2.77	83
3.33	2.73	82
6.00	4.81	80
Average..................................		85

100 mg. of creatinine.   Experiments were carried out to determine the conversion of creatinine into guanidines, hot alcohol being used to release the guanidine adsorbed on the norit.   The results are shown in Fig. 1, which is an interpolated graph of the average values obtained.   It would seem that the conversion is not strictly proportional to the concentration, but decreases with increased concentrations of creatinine.   For creatinine concentrations between 60 and 120 mg. per 100 cc., the average conversion value is approximately 0.70 mg. of methylguanidine per 100 mg. of creatinine (as observed by Weber), but even for this range the guanidines formed decrease with increased concentrations of creatinine.

The conversion of creatinine into guanidines by norit is apparently dependent upon several factors: the time of contact with the basic norit, the quantity of norit, the concentration of creatinine, the state of division of the norit, and the recovery of guanidines after they are formed.   In the method subsequently de-

scribed, the quantity of norit is constant, and the time of contact is kept as constant as possible. The state of division of the norit must vary with every sample and is probably one of the main inherent errors of the method. The concentration of creatinine is taken into account by the use of Fig. 1.

Conversion of Creatine into Guanidines by Norit—Although normal male urine contains no creatine, this substance occurs normally in the urine of children and sometimes in that of women, and also in various pathological disorders. Experiments showed that creatine also is converted into guanidines by basic norit.
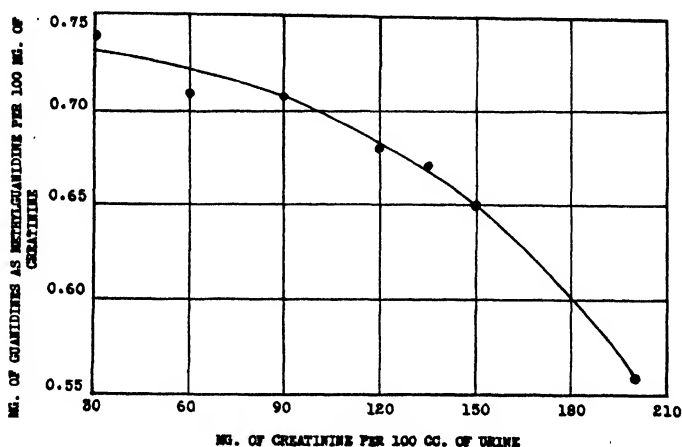
FIG. 1. Conversion of creatinine into guanidines in the method for guanidine determination in urine.

Table II shows that the conversion is also roughly proportional to the concentration of creatine, although, as with creatinine, the conversion decreases with increasing concentrations of creatine. The average value obtained is 3.1 mg. of guanidines per 100 mg. of creatine. This figure is only partly due to the conversion of creatine into guanidine, the major part being due to the color that creatine itself gives with the guanidine color reagent (creatine is extracted by norit along with the guanidines).

The use of the autoclave to convert the creatine into creatinine (as with the blood method) was not found advisable. Autoclaving caused a charring of the final product and also decreased the recovery of added guanidine compounds.

With creatine there is even more chance of variation than with creatinine. This could be predicted from the variations recorded in Table II. On this account, guanidine determinations on urines, containing much creatine, must be considered as little more than rough approximations.

Method

To 25 cc. of urine in a 50 cc. centrifuge tube are added 10 cc. of 40 per cent lead acetate and 5 cc. of 10 per cent sodium hydroxide. The contents of the tube are stirred and centrifuged. 30 cc. of the resultant supernatant liquid (unfiltered) are transferred

TABLE II

Influence of Creatine on Determination of Guanidine in Urine

Creatine added per 100 cc. of urine	Determined as methyl-guanidine per 100 cc.	Calculated as methylguanidine per 100 mg. of creatine
mg.	*mg.*	*mg.*
6.00	0.18	3.00
6.00	0.21	3.50
12.00	0.21	3.50
12.00	0.41	3.42
18.00	0.54	3.00
18.00	0.52	2.89
30.00	0.90	3.00
30.00	0.78	2.60
Average..		3.1

into another centrifuge tube, and 15 cc. of saturated disodium phosphate added to precipitate the surplus lead salt. The mixture is stirred and centrifuged as before.

8 cc. of the resultant solution are added to a 150 cc. Erlenmeyer flask, followed by 0.2 gm. (¼ teaspoonful) of norit.[1] The mixture is shaken for exactly 20 seconds and filtered through a suction filter with No. 2 Whatman filter paper. The flask is rinsed twice

[1] The norit is acid-washed. It is prepared as follows: To a suitable quantity of norit is added the same volume of 0.5 N HCl. The mixture is heated to boiling and filtered through a suction filter. The norit is washed while on the filter with several portions of distilled water. It is then removed from the filter and dried for 24 hours at 120°.

with distilled water containing sodium hydroxide (6 cc. of 10 per cent NaOH per liter), and the rinsings poured over the norit on the filter paper. The filter paper and norit are sucked dry and returned to the original flask. Then about 25 cc. of acid alcohol (2 cc. of 10 N HCl per liter of 95 per cent alcohol) are added immediately and the contents of the flask shaken.[2]

The contents of the flask are evaporated to dryness on a water bath kept between 80–90°. The evaporation is facilitated by drawing a current of air through the flasks. To the dry contents of the flask are added 30 cc. of 95 per cent ethyl alcohol, and the mixture is shaken well and filtered through a common filtering funnel (9 cm. Whatman filter paper). 25 cc. of the resultant filtrate are transferred to a 50 cc. Erlenmeyer flask, and evaporated to dryness on a water bath kept between 80–90° as before.

2 cc. of distilled water are added to the dry flask and standards made up containing 0.03, 0.04, and 0.05 mg. of methylguanidine[3] per 2 cc. of volume. (At least 4 cc. of standard are always prepared.) To both the unknown and the standards is added 0.5 cc. of the nitroprusside reagent[4] per 2 cc. of volume. The unknown solution is transferred to a small centrifuge tube and centrifuged for 3 minutes to remove the slight precipitate that always forms at this juncture. Colorimetric comparisons are made in a colorimeter equipped with microcups within 9 minutes from the time the reagent is first added. If a microcolorimeter is not available,

[2] The whole procedure from the time the material is in contact with norit in a basic solution until the acid alcohol is added should be completed as quickly as possible, owing to conversion of creatinine and creatine into guanidines.

[3] The methylguanidine standard is prepared by dissolving 167.1 mg. of methylguanidine sulfate in 100 cc. of distilled water. Dilutions of this standard in the ratio of 1:10 (and rarely 1:100) are the most convenient for use. The standard keeps perfectly in a refrigerator.

[4] The nitroprusside reagent is the one described by Weber (9). It is prepared by mixing the following: 1 part of 10 per cent sodium hydroxide, 1 part of 10 per cent sodium nitroprusside, 1 part of 10 per cent potassium ferricyanide, 9 parts of distilled water (NH_3-free). The reagent must stand at least 20 minutes after mixing before use. It need not be prepared previous to each determination, but will keep (mixed) for a long period of time if it is stored in brown bottles in a dark refrigerator. The slight precipitate that forms at the bottom of the bottle does not affect its value.

the amounts of material should be doubled throughout (and larger flasks used), so as to provide a larger amount of material for the color comparison.

Since the sample employed represents $3\frac{1}{3}$ cc. of urine, and five-sixths of this sample is present for the final determination, the guanidine is calculated by the following formula.

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{concentration of standard} \times 36 = \text{guanidine per 100 cc. urine}$$

Guanidine determinations are best made in duplicate, and the average of the two values taken as the result. The agreement of the two determinations should be good, unless creatine is present.

From the above value must be subtracted the correction for the conversion of creatinine into guanidines by the method. Although for dilute urines the correction can be taken as 0.70 mg. per 100 mg. of creatinine, the graph had best be employed (Fig. 1). In all cases, concentrated urines should be diluted to at least 1000 cc. before the determinations are made, since the creatinine corrections are not as reliable when the creatinine concentration is over 120 mg. per 100 cc.

If creatine is present, a correction must be applied for it also. As shown in Table II this correction amounts to about 3.1 mg. of methylguanidine per 100 mg. of creatine. As previously stated, when large amounts of creatine are present, the determinations of guanidine are erratic and can only be considered approximations.

Creatinine and creatine determinations are best made on the lead filtrate. Sufficient filtrate is present from the 25 cc. of urine for the creatine, creatinine, and duplicate guanidine determinations.

Guanidine Bases in Normal Urine

Table III gives the results of guanidine determinations on the urines of four normal male subjects. Three 24 hour specimens were analyzed for each person, the determinations being carried out in duplicate. The specimens were accurately collected at about 10 day intervals.

The results give the guanidine excretion of normal males as between 3 and 10 mg. per 24 hours. The average is about 6 mg., but even for each individual the values vary considerably. These values are about half those reported by Weber (8), which ranged between 10 and 20 mg. These lower values may be due to the lack of brown color in the final extracted material.

It is realized that the absolute accuracy of the values for guanidine reported are open to question, since they have been determined by difference, a procedure to be avoided where possible. The creatinine correction amounted to from 60 to nearly

TABLE III

Guanidine Bases in Normal Male Urine

Individual No.	Volume per 24 hrs.	Creatinine per 100 cc.	Creatinine correction	Guanidine determined per 100 cc.	Guanidine as corrected per 100 cc.	Guanidine per 24 hrs.
	cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	1060	214	1.18	1.96	0.78	8.27
	1490	165	1.02	1.64	0.62	9.24
	1320	187	1.08	1.84	0.72	9.50
2	1010	172	1.07	1.55	0.48	4.85
	860	254	1.25	2.00	0.75	6.90
	995	178	1.07	1.43	0.36	3.58
3	1820	130	0.87	1.34	0.47	8.55
	1135	194	1.11	1.42	0.31	3.52
	1510	122	0.83	1.25	0.42	6.34
4	1010	167	1.05	1.36	0.31	3.13
	1280	135	0.90	1.36	0.46	5.89
	1240	153	0.98	1.55	0.57	7.07

80 per cent of the total color value. However, since about 86 per cent recovery of added methylguanidine was obtained, and since a correction has been applied for creatinine, it seems probable that the substance determined is guanidine or some of its derivatives other than creatinine or creatine. It acts as guanidine or methylguanidine, but no direct evidence can be offered to show that it is either of these two compounds.

The values in Table III also show the guanidine-like substances in urine are only about 1½ to 4 times more concentrated than in normal blood. This is in marked contrast to creatinine, which is about 100 times more concentrated in urine than in blood.

SUMMARY

1. A modification of Weber's method for the determination of guanidine-like substances in urine is described. This method gives a final extract that is practically colorless. It also gives about 85 per cent recovery of added methylguanidine.

2. Tables are given for the conversion of creatinine and creatine into guanidines by the above method. These values show that the amount of guanidine formed by either creatinine or creatine is not strictly proportional to the concentration, but that the guanidines formed from a given amount of either creatinine or creatine decrease with increasing concentrations of these substances.

3. By this method, as modified, the guanidine-like substances excreted by four normal males were found to be between 3 and 10 mg. in 24 hours, the daily excretion varying even with each individual. These values are about 50 per cent lower than those given by Weber's method.

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AMINO ACIDS IN STAPLE FOODS*

I. WHEAT (TRITICUM VULGARE)

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Recent developments in the quantitative determination of amino acids provide suitable methods for the evaluation of staple foods based on their ability to supply certain nutritionally indispensable amino acids. In the past a large number of proteins have been isolated from most of our commonly used food materials, and their amino acid composition has been determined. Little is known, however, regarding the relative proportions of the different proteins present in these food materials; consequently there is no basis upon which the amount of any given amino acid in the food material as a whole can be estimated. Furthermore, to estimate the amino acid content of staple foods from the data on individual proteins as found in the literature would be inaccurate, since amino acids are present in foods in a free state or in combination as peptides, hormones, etc. Direct determination of the nutritionally indispensable amino acids will find practical application in adjusting and correcting amino acid deficiencies in foods by proper supplementation and also in selecting for planting the variety of seed shown to be richest in indispensable amino acids.

EXPERIMENTAL

The simplest and most direct way to determine amino acids in wheat is to liberate them by acid hydrolysis from any combination that may exist in the kernel; but experience has shown that such

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* The material in this paper was presented before the Twenty-ninth and the Thirtieth meetings of the American Society of Biological Chemists (*Proc. Am. Soc. Biol. Chem.*, **8**, xxv (1935); **8**, xxiii (1936); *J. Biol. Chem.*, **109** (1935); **114** (1936)).

a procedure involves losses and destruction for some of them (1). The next choice is to separate the nitrogenous material by using suitable solvents previous to their hydrolysis. Sulfuric acid as a hydrolyzing agent has the advantage over HCl in that by the use of Ba one can easily free the hydrolysate from it. Starch can be dispersed, however, in cold 20 to 21 per cent HCl and reprecipitated by alcohol as shown by Rask (2), and the use of HCl has the further advantage that on concentration it reaches 20 to 21 per cent, which is the strength desired for hydrolyzing proteins.

Through the kindness of Dr. Coleman of the Bureau of Agricultural Economics, United States Department of Agriculture, whole wheat flour was prepared from cleaned and selected whole wheat kernels of a known single variety. To obtain a hydrolysate in which the amino acids, cystine, histidine, arginine, and lysine, may be determined the following technique was found satisfactory. 40 gm. of the freshly ground whole wheat flour transferred into a 500 cc. centrifuge bottle are defatted by three $\frac{1}{2}$ hour extractions with ether at room temperature. Longer contact of ether with the protein material lowers the yield of N in the salt extracts. The defatted material is then extracted with a 1 per cent aqueous NaCl solution by the addition of 100 cc. of the precooled solvent and occasional shaking. Three of these salt extractions of 1 hour's duration and at refrigeration temperature (6–8°) are sufficient to remove the water and salt-soluble nitrogenous substances. Three 60 per cent aqueous alcohol extractions in 100 cc. portions and at room temperature follow, the first two of $1\frac{1}{2}$ to 2 hours duration each and the third one overnight. These are followed by the acid-alcohol extraction. 100 cc. of a cold 20 per cent HCl solution are added, and the mixture is placed in the refrigerator for 1 hour and stirred occasionally to facilitate the dispersion of starch and to allow a better extraction of the nitrogenous substances. At the end of the hour, 150 cc. of 95 per cent alcohol are added slowly with constant stirring. Then the mixture is centrifuged and the liquid is set aside. This acid-alcohol extraction procedure is repeated.

The denatured protein material which did not go into the acid-alcohol extract is freed from starch by the addition of cold 20 per cent HCl; one 300 cc. portion followed by a 100 cc. portion is generally sufficient to accomplish that. The residue is then hydrolyzed in 20 per cent HCl for 24 hours.

In Table I the nitrogen contents of the several extracts from three varieties of wheat are given to show the yield and efficiency of the technique described above. The nitrogen is determined by the Kjeldahl method separately in the salt-alcohol and acid-alcohol extracts on 0.02 aliquots in duplicates. The salt and alcoholic extracts are evaporated to a small volume on the water bath, in a large porcelain casserole, then transferred into a 300 cc. round bottom Pyrex flask. The coagulum is scraped off with a glass spatula, and the dish is rinsed with a few cc. of 20 per cent HCl as quantitatively as possible. The acid-alcohol extract is now evaporated to approximately 20 cc., in the same casserole, and combined with the previous concentrate 20 per cent HCl, and finally a few cc. of distilled water are used to accomplish a quantita-

TABLE I

Nitrogen Extracted by Various Solvents, Indicated and Expressed in Percentage of Total Wheat Nitrogen

Solvent	Wheat varieties		
	Tenmarq	Marquis	Fulhio
1% NaCl	24.4	24.0	26.8
60% alcohol	32.0	38.5	32.1
Acid-alcohol	20.5	14.5	15.8
Residue hydrolysate	18.8	15.0	19.2
Total yield	95.7	92.0	93.9

tive transfer. For 1 hour or so the combined extracts are heated in an oil bath at 120–125° to bring the volume approximately to 40 cc. and at the same time to let the HCl adjust itself to a 20 per cent concentration. If the volume is lower than 40 cc., 20 per cent HCl is added to bring it to that volume. By placing a Hopkins condenser on the flask the material is hydrolyzed for 24 hours. The hydrolysate is filtered by suction, and the humin precipitate washed thoroughly with hot 0.1 N HCl. This can be accomplished best by returning the humin into the flask with the aid of a glass spatula, leaving the suction on to keep the filter paper in a fixed position. The final volume of the hydrolysate is kept around 80 cc. The hydrolysate of the residue is filtered also, and the humin is washed as stated above.

Determination of Amino Acids. Cystine—Sullivan's method (3) was applied with certain modifications. With Bürker's colorimeter it was found unnecessary to use decolorizing agents of any sort. 1 to 2 cc. of the hydrolysate matched a suitable cystine color standard. Duplicate samples were taken, to one of which concentrated NaOH solution was added until the last drop changed the blue color of a Congo red paper to reddish blue; then sufficient distilled water was added to bring the volume up to 5 cc. This sample was used in the compensator cup; the same amount of reagents were added to it later as to the unknown, except that the naphthoquinone sodium sulfonate was omitted. To the second sample the volume of distilled water ascertained above was added and then alkali was added drop by drop, the flask being kept in a cool water bath and agitated continuously. The cystine color test was carried out from this point according to Sullivan's method.[1] Hydrolysate of the residue contained a very small quantity of cystine (or none) and, therefore, it was necessary to use a larger (5 cc.) sample.

Basic Amino Acids—A known volume of the remaining hydrolysate is concentrated to sirupy consistency *in vacuo* to drive off the excess of HCl. This procedure is repeated twice after the addition of 100 cc. of distilled water to each one, and finally the hydrolysate is transferred to a 500 cc. Pyrex centrifuge bottle. A corresponding aliquot of the hydrolysate of the residue is treated similarly to remove excess HCl and then combined with the previous one. Concentrated silver nitrate solution is added in slight excess to remove chlorine quantitatively, then centrifuged, and the precipitate is washed twice with approximately 50 cc. of distilled water. The second time 5 cc. of 0.1 N HCl are added to the water to insure the quantitative removal of histidine and arginine. The washing is then repeated twice with hot acidified water. The combined liquids are concentrated *in vacuo* to a small (30 cc.) volume. Silver nitrate and a few drops of sulfuric acid are added to remove chlorine. The mixture is then centrifuged, and the supernatant liquid is filtered into a 100 cc. centrifuge bottle. More silver nitrate is added to insure the presence of free Ag ion. The final volume should not be larger than 50 cc. After this, the procedure

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[1] According to Dr. Sullivan's advice the sodium cyanide was dissolved in 0.5 N sodium hydroxide solution instead of water.

described in a previous communication (1) for the basic amino acid determination was followed. A correction was made, however, in regard to the volume of the histidine concentrate, which should read 10 cc. instead of 25 when Hopkins' reagent is added. Furthermore, the lysine phosphotungstate precipitate should not be heated but centrifuged off after standing overnight in a refrigerator. The precipitate should be redissolved in 25 to 30 cc. of hot 2.5 per cent phosphotungstic acid in 5 per cent sulfuric acid, and left in the refrigerator for 3 to 4 hours. By heating the phosphotungstate in the washing solution instead of the hydrolysate concentrate, there is less danger in losing lysine, and also the removal of impurities is more efficient.

A correction of 0.04 was made in the amino acid quantities, for

TABLE II

*Amino Acid Content and Total Nitrogen in Percentages of Moisture-Free Wheat**

Variety of wheat and place grown	Cystine	Tryptophane	Tyrosine	Arginine	Histidine	Lysine	Total N
Marquis, hard spring; North Dakota........ ..	0.270	0.090	0.866	0.510	0.280	1.510	3.31
Tenmarq, hard winter; Kansas................	0.170	0.066	0.560	0.376	0.117	1.225	2.58
Fulhio, soft winter; Ohio...	0.157	0.080	0.405	0.356	0.080	0.872	2.34

* The figures represent averages of duplicate analyses.

the amount removed for the determination of nitrogen in the several extracts. Since the amino acids mentioned above did not contribute to humin formation, we felt justified in disregarding the humin nitrogen formed during hydrolysis, and values given in Table II may well be considered absolute values.

Tryptophane—May and Rose's method with slight modification for tryptophane, as given in a previous communication (4), was applied with 0.6 gm. of material. It was found advantageous to filter the casein standard and the unknown solutions before they were compared in the colorimeter. The presence of starch was found to influence the accuracy, but the loss was slight. (Less than 5 per cent was observed when 0.5 gm. of starch was added to a casein standard.)

Tyrosine—The method of Folin and Ciocalteu (5) was used with 2 gm. of material.   The use of Bürker's colorimeter with its color-compensating feature proved to be a great improvement here as well as in the cystine determination mentioned above.   The unknown sample for the compensating cup was prepared similarly and simultaneously with the other one, except at the end the addition of sodium nitrate solution was omitted.

<center>DISCUSSION</center>

Certain amino acids required in the synthesis of body proteins and hormones must be available to the animal through the food supply.   It would be more logical, therefore, if in the future the protein-furnishing staple foods would be rated according to their

<center>TABLE III</center>

Protein Quality As Shown by Relationship between Amino Acids and Total Nitrogen Content

Mg. of indicated amino acid per gm. of total nitrogen.

Name	Cystine	Tryptophane	Tyrosine	Arginine	Histidine	Lysine
Marquis	81	28	262	154	85	456
Tenmarq	65	26	173	146	43	475
Fulhio	67	34	217	152	34	373
Casein for comparison	20	130	405	236	156	475

amino acid content with emphasis placed on the indispensable ones rather than solely on the nitrogen content.   The nitrogen content is still a convenient guide to estimate the quantity of protein present, but when malnutrition sets in, pointing to amino acid deficiency, the knowledge of the amino acid composition will lead to a remedy for the existing deficiency.

The amino acid percentages given in Table II show that the amino acid content is different in the different varieties of wheat studied.   Hard wheat is generally richer in protein than soft wheat, and the protein quality is better in the varieties with higher protein content.

When the quantities of amino acids of whole wheat are calculated per gm. of wheat nitrogen and compared with casein, as shown in Table III, it is found that the tryptophane content of

wheat protein is rather low.   Consequently a diet in which wheat is the only source of protein is liable to be deficient in tryptophane.

SUMMARY

The individual amino acid content varies in different wheat varieties.   The protein quality is much better in wheat varieties of higher nitrogen content than in those of low nitrogen.   The nutritive value of the protein of whole wheat flour compares favorably with that of casein; among the indispensable amino acids studied only tryptophane is present at a low level.

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STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

III. THE EXTENT OF THE SYNTHESIS OF *p*-BROMOPHENYLMER-CAPTURIC ACID IN DOGS AS RELATED TO THE TIME OF ADMINISTRATION OF FOOD AND BROMOBENZENE

By JAKOB A. STEKOL

With the Technical Assistance of John R. Foy

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Foresgren (1) has shown that in rabbits the activity of the liver is periodic in character. He observed that maximum deposition of glycogen in the liver took place at night, reaching a minimum during the daytime. He also learned that the secretion of bile alternated with the formation of glycogen, and that these two functions were independent of food consumption. Jorpes and coworkers (2) observed these cyclic changes in the livers of mice and rats. Higgins and coworkers (3) noted a bimodal cycle in the changes of the weight of the livers of chickens, rabbits, and guinea pigs during the digestive phase. These workers, however, concluded that the glycogen, water, and protein content of the liver closely follows the changes in the weight of the liver, being apparently dependent on the food and water consumption.

It is believed that the extent of the synthesis of *p*-bromophenyl-mercapturic acid in animals depends on the immediate supply of dietary sulfur. When the animal is deprived of sulfur in the diet or when the sulfur of the diet is too low to meet the needs for detoxication of bromobenzene, the animal utilizes its tissue sulfur for the purpose. If, however, the sulfur in the form of protein or *l*-cystine or *dl*-methionine is supplied by the diet, the sulfur-containing amino acids are utilized in the detoxication processes in preference to the tissue sulfur (4). It follows from the above theory that the degree of toxicity of bromobenzene and the extent of the synthesis of *p*-bromophenylmercapturic

acid should depend on the time of administration of bromo-
benzene as related to the time of administration of food, since
it is upon the availability of food sulfur that the mode of detoxica-
tion of bromobenzene depends.   Thus, if the animal is fed once
a day at 9 a.m. and bromobenzene is administered at 9 p.m.,
greater toxicity and less efficient detoxication should result, as
compared to the results obtained when the animal is fed at 9 a.m.
and bromobenzene is administered also at 9 a.m.

Assuming that the detoxication of bromobenzene takes place
in the liver, it occurred to us that should the dog liver possess
cyclic activity with respect to detoxication capacity, analogous to
that observed with glycogen deposition and bile secretion (1),
feeding of bromobenzene at various times of the 24 hour period
should result in variable yields of the detoxication products of
bromobenzene in the urine.   We therefore investigated the extent
of the detoxication of bromobenzene in dogs, with reference to
the time of administration of food and bromobenzene.

EXPERIMENTAL

Adult female dogs were used exclusively.   The plan of the ex-
periments was to feed the dogs Cowgill's diet (5) once a day at
9 a.m., collect the urine by catheterization just before feeding
and then again at 9 p.m., and analyze each 12 hour portion of
urine for various constituents until the values showed little or
no variation from day to day; to feed a 1.0 gm. single dose of
bromobenzene at 9 a.m. and continue the collection of urine and
the analysis as before until the values had returned to normal;
then to repeat the feeding of 1.0 gm. of bromobenzene at 9 p.m.
instead of at 9 a.m.   The same dogs were then fed the same diet
in the same amount at 9 p.m. once a day and bromobenzene was
administered again, in a 1.0 gm. dose, at 9 a.m. and then, in
another experiment, at 9 p.m. p-Bromophenylmercapturic acid
was estimated in each 12 hour sample of urine by the recently
described method (6) until the urine showed the absence of mer-
capturic acid.   Other urinary constituents were determined by
methods which were previously described (7).   Cowgill's diet
(5) contained 43.7 parts of casein, 40.6 of sucrose, 11.6 of Squibb's
vitavose, 1.4 of Karr's (8) salt mixture, 2.7 of bone ash, 17 of
lard, and 7 parts of butter fat.   The diet yielded 4.80 per cent

of nitrogen and 0.248 per cent of sulfur. Bromobenzene was redistilled before use.

DISCUSSION

For the sake of economy of space the results on only one dog are presented in Tables I and II. Similar data were obtained with the other two animals.

TABLE I

Metabolism of Bromobenzene in Dogs As Related to Time of Its Administration

Dog 14, weight 9.0 kilos. Food at 9 a.m.; urine collected at (a) 9 a.m. to 9 p.m. and (b) 9 p.m. to 9 a.m. Intake, 4.80 gm. of nitrogen and 0.248 gm. of sulfur.

Day No.	Period	Total N	Urea N	Creatinine	Total S	Inorganic SO₄-S	Ethereal SO₄-S	Neutral S	Mercapturic acid	
									Per 12 hrs.	Total
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	a	2.80	2.20	0.109	0.122	0.070	0.011	0.041		
	b	1.39	1.09	0.095	0.080	0.044	0.008	0.028		
2	a	2.77	2.29	0.102	0.133	0.088	0.012	0.033		
	b	1.46	1.08	0.099	0.076	0.039	0.008	0.029		
3*	a	2.41	1.86	0.099	0.141	0.031	0.028	0.082	0.393	
	b	1.75	1.43	0.097	0.078	0.004	0.019	0.055	0.253	
4	a	2.65	2.25	0.092	0.083	0.019	0.016	0.048	0.105	
	b	1.60	1.21	0.081	0.075	0.027	0.009	0.039	0.083	0.834
5	a	2.39	2.07	0.097	0.127	0.082	0.011	0.034		
	b	1.83	1.41	0.090	0.083	0.037	0.008	0.037		
6	a	2.66	2.11	0.113	0.140	0.086	0.012	0.042		
	b	1.64	1.18	0.095	0.073	0.039	0.007	0.027		
7	a	2.70	2.10	0.110	0.129	0.079	0.011	0.039		
	b	1.60	1.20	0.096	0.060	0.027	0.008	0.025		
8†	a	2.46	1.91	0.095	0.107	0.061	0.011	0.035		
	b	1.77	1.31	0.082	0.122	0.018	0.029	0.075	0.464	
9	a	2.73	2.25	0.106	0.100	0.021	0.019	0.060	0.190	
	b	1.68	1.31	0.086	0.068	0.016	0.010	0.042	0.133	
10	a	2.91	2.40	0.094	0.127	0.075	0.010	0.042	0.090	0.877
	b	1.38	1.02	0.078	0.072	0.025	0.008	0.039		

* 1.0 gm. of bromobenzene was fed at 9 a.m.

† 1.0 gm. of bromobenzene was fed at 9 p.m.

The data presented show that whether the food and bromobenzene are fed simultaneously at 9 a.m. or at 9 p.m. or separately, food at 9 a.m. and bromobenzene at 9 p.m., or *vice versa,*

the extent of the synthesis of p-bromophenylmercapturic acid and of ethereal sulfates is about the same in all cases. It appears that whether or not the activity of the liver in dogs is cyclic in character, as regards the deposition of glycogen and secretion of bile, the capacity of the animal to synthesize p-bromophenyl-

TABLE II

Metabolism of Bromobenzene in Dogs As Related to Time of Its Administration

Dog 14, weight 9.0 kilos. Food at 9 p.m.; urine collected at (*a*) 9 a.m. to 9 p.m. and (*b*) 9 p.m. to 9 a.m. Intake, 4.80 gm. of nitrogen and 0.248 gm. of sulfur.

Day No.	Period	Total N	Urea N	Creatinine	Total S	Inorganic SO_4-S	Ethereal SO_4-S	Neutral S	Mercapturic acid Per 12 hrs.	Mercapturic acid Total
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	*a*	1.33	0.99	0.087	0.075	0.028	0.015	0.032		
	b	3.05	2.48	0.106	0.139	0.093	0.007	0.039		
2	*a*	1.40	1.09	0.087	0.068	0.030	0.010	0.028		
	b	2.86	2.28	0.105	0.142	0.101	0.009	0.032		
3*	*a*	1.46	1.09	0.088	0.112	0.016	0.023	0.073	0.364	
	b	2.99	2.54	0.098	0.098	0.016	0.018	0.064	0.261	
4	*a*	1.41	1.08	0.081	0.065	0.010	0.012	0.043	0.150	
	b	2.91	2.50	0.091	0.128	0.077	0.010	0.041	0.123	0.898
5	*a*	1.40	1.00	0.099	0.073	0.031	0.008	0.034		
	b	3.03	2.50	0.107	0.146	0.097	0.006	0.043		
6	*a*	1.37	1.00	0.093	0.064	0.024	0.010	0.030		
	b	3.02	2.58	0.105	0.145	0.105	0.006	0.034		
7†	*a*	1.50	1.18	0.084	0.070	0.030	0.007	0.031		
	b	3.12	2.49	0.099	0.166	0.045	0.031	0.090	0.352	
8	*a*	1.44	1.00	0.095	0.070	0.002	0.018	0.050	0.229	
	b	3.06	2.40	0.105	0.102	0.044	0.013	0.045	0.146	
9	*a*	1.40	1.01	0.096	0.083	0.030	0.013	0.040	0.090	0.817
	b	3.04	2.50	0.106	0.141	0.103	0.008	0.030		

* 1.0 gm. of bromobenzene was fed at 9 a.m.

† 1.0 gm. of bromobenzene was fed at 9 p.m.

mercapturic acid and the ethereal sulfates from bromobenzene is independent of such a rhythmic cycle. Although there is some evidence to show that certain detoxication reactions take place in the liver (9), it remains to be demonstrated that p-bromo-phenylmercapturic acid (and other mercapturic acids) is syn-

thesized by the liver. Whether or not the liver is the seat of mercapturic acid formation in dogs, the extent of such a synthesis is apparently independent of the time of day at which either the bromobenzene or the food is fed to well nourished animals.

The creatinine output in the urine did not seem to be affected by the administration of bromobenzene. A similar observation was made even when bromobenzene was fed to fasting animals (10). This would indicate that the utilization of tissue sulfur for the detoxication of bromobenzene is not necessarily accompanied by an increase in the creatinine output in the urine. Whether the food was fed at 9 a.m. or at 9 p.m., feeding of bromobenzene at 9 a.m. or at 9 p.m. invariably caused an increased output of total sulfur in the urine, which was compensated by a retention of dietary sulfur during the period following ingestion of food. The excretion of total nitrogen, however, remained practically unaffected throughout the experiments. This observation is similar to the one reported in Paper I (11). Our data suggest that the dietary sulfur is not apparently the immediate source of sulfur which is utilized in the detoxication of bromobenzene in dogs. The rise in total sulfur upon administration of bromobenzene to dogs probably indicates the utilization of sulfur other than that of the diet; the apparent constancy of urinary nitrogen and the retention of dietary sulfur suggest the resynthesis of the attacked tissue in the presence of an adequate supply of sulfur in the diet.

SUMMARY

1. Adult dogs were fed Cowgill's diet once a day, either at 9 a.m. or at 9 p.m. A 1.0 gm. single dose of bromobenzene was fed either at 9 a.m. or at 9 p.m. under each dietary condition and the extent of the synthesis of p-bromophenylmercapturic acid was estimated.

2. The extent of the synthesis of p-bromophenylmercapturic acid in well nourished dogs seems to be independent of the time of administration of either the food or of the bromobenzene.

3. The results obtained are in line with our previous suggestion that the dietary sulfur is not the *immediate* source of sulfur which is utilized in the detoxication of bromobenzene.

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NOTE ON TURBIDITY IN URIC ACID DETERMINATIONS, WITH SPECIAL REFERENCE TO THE PHOTO-ELECTRIC COLORIMETER*

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The determination of uric acid by the Benedict methods (1–3) with a photoelectric colorimeter such as the one suggested by the writer (4) requires some precautions which are not often apparent when the visual instrument or photoelectric colorimeters which match unknown with standard are used.

Turbidity is reported to have troubled many analysts. The writer has found that this may be overcome by increasing the concentration of the hydrochloric acid in the standard. The methods (1, 2) call for the presence of hydrochloric acid in the standard solution to the extent of 1 part of hydrochloric acid, sp.gr. 1.19, in 200 parts of standard solution. This concentration of hydrochloric acid is the critical limit at which turbidity will be prevented, while no undesirable effects occur if the concentration of hydrochloric acid is several times this amount. However, a larger excess of acid is to be avoided as it will fade or completely bleach the solution.

This is of particular importance when making transmission-concentration calibration curves for photoelectric colorimeters. These curves are prepared by diluting differing amounts of standard to the same volume. Then the same quantities of color reagents are added to all the known solutions. When the amount of standard is less than the prescribed amount (5 cc. in the blood methods (1, 3) and 10 cc. in the urine method (2)), turbidity will occur unless *the diluent contains the same concentration of hydrochloric acid as the original standard.* Thus in the blood methods

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(1, 3) the dilution to the prescribed 5 cc. is made with 1 part of hydrochloric acid, sp.gr. 1.19, to 200 and 100 parts of water respectively and in the urine method (2) the dilution to the prescribed 10 cc. is made with 1:200 hydrochloric acid. In no event should dilution be made after the color reagents have been added.

It is also important that the time required for the uric acid color to develop be carefully controlled (interval timer). The time factor can easily be studied with the photoelectric colorimeter. Table I shows the effect of time on the variation of depth of color of a typical uric acid (urine) solution. A Corning No. 294 red

TABLE I

Effect of Time on Variation of Depth of Color of Typical Uric Acid Solution

Time*	Transmission	Equivalent concentration
min.	*per cent*	*cc. standard*
0	14.0	10.2
10	12.7	10.7
20	11.3	11.3
30	10.7	11.6
60	10.0	12.0

* Time after development of the color as specified in the Benedict method (2).

filter was used. The curves for this group of determinations show appreciable blanks, and a change in the color reagents may alter the position of the origin of the curve, but will not alter its slope.

The writer's thanks are due Dr. K. G. Falk and Miss Grace McGuire for their untiring advice.

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THE DENATURATION AND HYDRATION OF PROTEINS*

II. SURFACE DENATURATION OF EGG ALBUMIN

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In Paper I of this series we (1) investigated the water- and ethyl alcohol-binding capacity of heat- and surface-denatured protein. These studies did not concern themselves with the mechanism of denaturation, but rather with the structure of the denatured products. In the present paper we have endeavored to study the kinetics of surface denaturation and to gain some insight into the mechanism of the process. Although there have been many such investigations of heat denaturation (*cf.* Chick and Martin (2) and Pauli and Weissbrod (3)), but little work has been reported on the mechanism of surface denaturation. A thorough study of this process should be of importance, not only since it would afford a comparison between these two kinds of denaturation, but also because it should throw light upon the physical chemistry and biochemistry of protein systems. Thus, the phenomenon of the formation of monomolecular protein films (4), on the one hand, and the problem of the formation of membranes of living cells, on the other, seems to be based on surface denaturation. As long ago as 1840, Ascherson (5) recognized the possibility that cell surfaces are made up in part, at least, of surface-denatured protein and, more recently, Gortner (6) comments on this idea:

"Such a mechanistic picture of the plasma membranes would probably consist of a more or less completely denatured (surface-energy coagulated) protein gel, probably in the form of a fibrillar structure with fats, soaps,

and lipides immeshed in the protein net-work. The transfer of lipide-soluble material would be through the fat-soap-lipide portion of the structure, whereas the passage of water and such water-soluble materials as actually do pass in and out of the cells would be through the hydrated filaments of the protein net-work."

In the present paper we have investigated the mechanism of surface denaturation by means of the following criteria: (1) the influence of protein concentration on the rate of denaturation; (2) the influence of pH on denaturation; (3) the influence of electrolytes on denaturation; (4) the influence of surface-active material on denaturation.

Preparation of Material—Two methods were used for the preparation of isoelectric, salt-free ovalbumin solutions. Ovalbumin I was prepared as described in Paper I (1); *i.e.*, by electrodialyzing fresh diluted egg white which previously had been freed from most of the globulins. This preparation yielded a 4.20 per cent solution with pH 4.85 and an electrical conductivity of 1.12×10^{-5} mho. Ovalbumin II was prepared by repeated crystallizations (three times) with ammonium sulfate and sulfuric acid, according to the Sørensen technique (7). The final crystals were dissolved in distilled water and dialyzed against distilled water to the absence of sulfate ions. The albumin solution was then evaporated to dryness below 50°. The dried product so obtained was dissolved again in water and electrodialyzed to a conductivity of 1.18×10^{-5} mho. This sol had a protein content of 1.75 per cent and a pH of 4.88. The purified solutions were covered with a thin layer of a paraffin-toluene mixture and stored until used at 2°.

Methods

A measured quantity of the protein solution was put in wide mouth glass-stoppered bottles of 70 cc. volume (approximately 7×3.5 cm. internal measurement) and shaken in a shaking machine in a horizontal position in an air thermostat at 2°. The rate of shaking was maintained constant, *i.e.* 96 movements per minute with a 7 cm. stroke. In some cases a higher rate of 140 movements per minute was employed, other factors being kept constant. After the elapsed time, the bottles were removed from the shaking machine, the precipitated protein was filtered off, and the protein content of the filtrate determined. If no electrolyte

other than HCl or ammonia was present, the concentration was determined by drying a given volume at 108° to constant weight. If electrolytes were present, but no ammonia, the protein concentration was determined by Kjeldahl analysis. All determinations were carried out in duplicate. In the later work, the amount of denatured protein was found by washing the precipitated protein four times with distilled water in a centrifuge tube, filtering through a sintered glass filter, and drying at 108° to constant weight. This method was found to yield exactly the same values as the earlier methods. In the first part of this investigation, pH measurements were made by means of the quinhydrone electrode, but in the last part they were made with a glass electrode.

EXPERIMENTAL

The dependence of the rate of surface coagulation on concentration is shown in Table I. Three different concentrations of Ovalbumin I were used, *viz.* 2.10, 1.10, and 0.55 per cent. In certain experiments some Pyrex glass beads were added to the solution in order to increase the force of breaking the surface by shaking; in two out of three cases the amount coagulated increased slightly.

From the last column of Table I it is seen that the limiting value of the coagulable protein is, with all concentrations investigated, between 73 and 79 per cent of the original concentration. Even 48 hours shaking of a 2.10 per cent solution did not convert more than 73 per cent of the protein into a surface-coagulated product. When the filtrate from this experiment was shaken again (25 cc., 24 hours), no further precipitation occurred. An analysis of Ovalbumins I and II with regard to distribution of surface-coagulable, heat-coagulable, and non-coagulable protein is shown in Table II. The material in the filtrate from the heat coagulation apparently contained no true protein, since it gave no reaction with sulfosalicylic acid.

The data in Table II indicate not only that electrodialysis alone is not sufficient to purify ovalbumin completely, but also that a small amount of heat-coagulable protein cannot be precipitated by surface denaturation. Table II also shows that with Ovalbumin II (crystallized and electrodialyzed) the non-coagulable portion amounts to only 4 per cent of the total concentration, indicat-

ing that crystallization is a more suitable method of purification. It would seem preferable to refer the amount coagulated, not to the initial concentration but to the "active concentration," *i.e.*,

TABLE I

Influence of Concentration on Rate of Surface Coagulation of Ovalbumin I

c = total concentration of protein in solution; c' = concentration of surface-denaturable protein in solution.

Time of shaking	Amount of protein coagulated (x)	Protein coagulated (x/c)
hrs.	*gm.*	*per cent*
$c = 2.10\%$; $c' = 1.53\%$. Rate of shaking 96 oscillations		
4.5	0.39	20.5
13	0.86	41.23
13*	0.95*	45.0*
18	1.14	54.5
18*	1.21*	57.8*
36	1.47	70.7
44	1.52	72.5
44*	1.50*	71.6*
$c = 1.10\%$; $c' = 0.86\%$. Rate of shaking 96 oscillations		
3	0.38	32.7
4	0.34	30.9
5.25	0.44	41.0
12	0.83	75.5
18	0.83	75.5
20	0.86	78.0
28	0.85	76.9
48	0.86	78.0
$c = 0.55\%$; $c' = 0.44\%$. Rate of shaking 96 oscillations		
1	0.14	25.5
4.5	0.44	79.9
8.75	0.43	78.2
18	0.44	79.9

* Pyrex glass beads were added to the solution in order to increase the force of breaking the surface.

the maximum amount coagulable by shaking (the c' of Table I). Fig. 1 (Curves 1 to 3) illustrates the recalculation of the data in Table I on such a basis. The velocity constants indicated on the curves were obtained from the slope of the fairly straight lines

TABLE II

Distribution of Surface-Coagulable, Heat-Coagulable, and Non-Coagulable Protein in Ovalbumin I and Ovalbumin II

Time of shaking 48 hours.

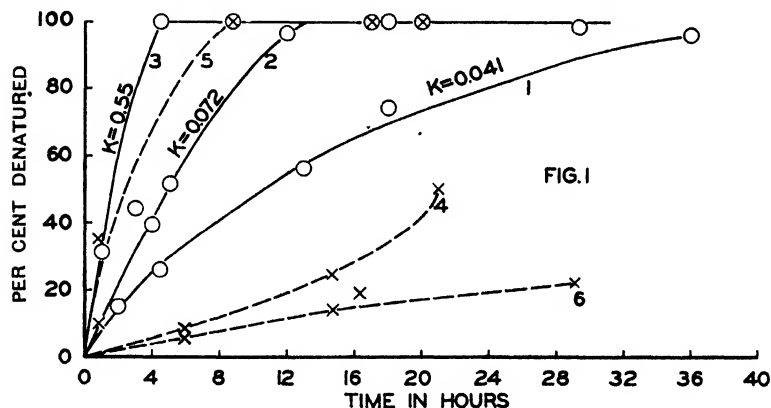|	Protein concentration	Surface-coagulable	Heat-coagulable	Non-heat-coagulable
	per cent	per cent of total	per cent of total	per cent of total
Ovalbumin I	2.15	72.6	83.8	16.2
	2.15			14.2
	1.10	78.2	81.8	18.2
	0.98			17.3
	0.55	78.2	80.0	20.0
Ovalbumin II	1.77	89.3	95.7	4.3
	1.22	91.8	95.7	4.3

FIG. 1. Showing the extent of surface denaturation as a function of time, all values being calculated on the basis of total coagulable protein. Curve 1, 25 cc. of a 2.10 per cent solution shaken at the rate of 96 oscillations per minute; Curve 2, 25 cc. of a 1.10 per cent solution shaken at the rate of 96 oscillations per minute; Curve 3, 25 cc. of a 0.55 per cent solution shaken at the rate of 96 oscillations per minute; Curve 4, 10 cc. of a 1.10 per cent solution shaken at the rate of 96 oscillations per minute; Curve 5, 10 cc. of a 1.10 per cent solution shaken at the rate of 140 oscillations per minute; Curve 6, 50 cc. of a 1.10 per cent solution shaken at the rate of 96 oscillations per minute. The values indicated for K are the velocity constants for the surface denaturation reaction at the different protein concentrations.

found when time was plotted against log $(c')/(c' - x)$ where c' is the initial concentration of coagulable protein and x is the amount coagulated.

The dependence of surface coagulation on pH is shown in Fig. 2. The pH was adjusted by adding HCl or ammonia, respectively, to the protein solution, the protein concentration being kept constant. At pH 1.06 and 9.156 a slight turbidity of the original solutions
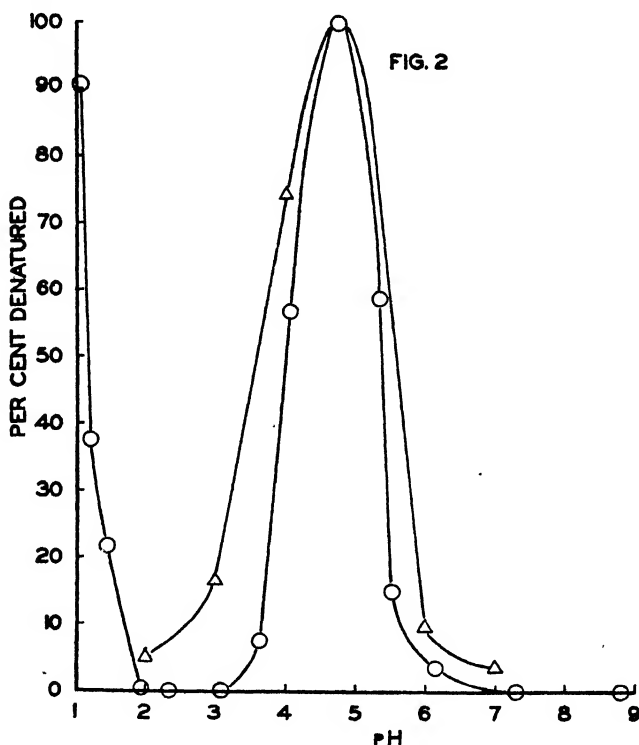

FIG. 2. Showing the extent of surface denaturation of a 1.05 per cent solution of egg albumin after 12 hours shaking at several pH values in the absence of electrolyte other than the HCl or ammonia necessary to secure the desired pH. All values are calculated on the basis of the total coagulable protein. ○, solutions analyzed for the amount of protein precipitated at any given pH; △, solutions brought back to the isoelectric point before analysis and the total amount of denatured protein obtained.

was observed, due to denaturation by the strong acid and base. In all cases the time of shaking was 18 hours with a protein concentration of 1.05 per cent. The upper curve is the amount of denatured protein found by bringing the solutions to the isoelectric point before analysis.

The change of pH which is brought about by surface coagulation is illustrated in Fig. 3, in which the ΔpH values are plotted as ordinates and the pH of the solution before denaturation began as abscissæ. The similarity between this curve and that obtained by Pauli and Koelbl (8) for the change of pH during heat denaturation is shown by the insert curve which is plotted in the upper right-hand corner of Fig. 3 against the same coordinates.
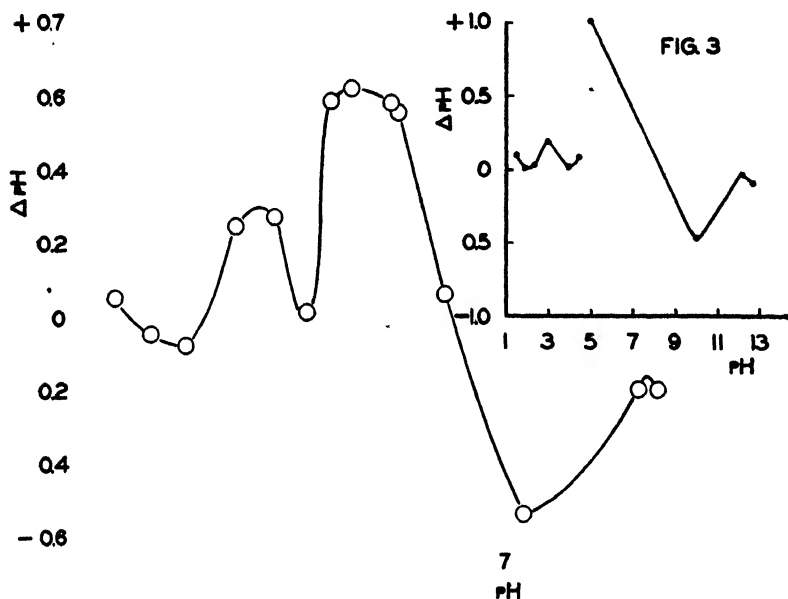

Fig. 3. Showing the change of pH of a 1.05 per cent protein solution after 18 hours shaking at several pH values. The insert in the upper right-hand corner shows similar data of Pauli and Koelbl for heat denaturation of egg albumin.

The addition of small amounts of inorganic salts had no effect on the rate or extent of surface denaturation and it was only when the salt concentration had been increased to 0.01 N or higher that there was any change in the rate of denaturation of the protein at the isoelectric point. 1.0 N KCl and 0.5 N K_2SO_4 increased the rate somewhat, while 0.5 N KSCN decreased it. 0.1 M sucrose tended to increase the rate of denaturation slightly, while 0.1 M

urea was without effect.[1]   Gelatin in all concentrations up to the gelling point was found to be without effect.

The action of 0.01 N K_2SO_4, 0.01 N $BaCl_2$, and 0.01 N KCl on the rate of denaturation of a 1.05 per cent protein solution was investi-
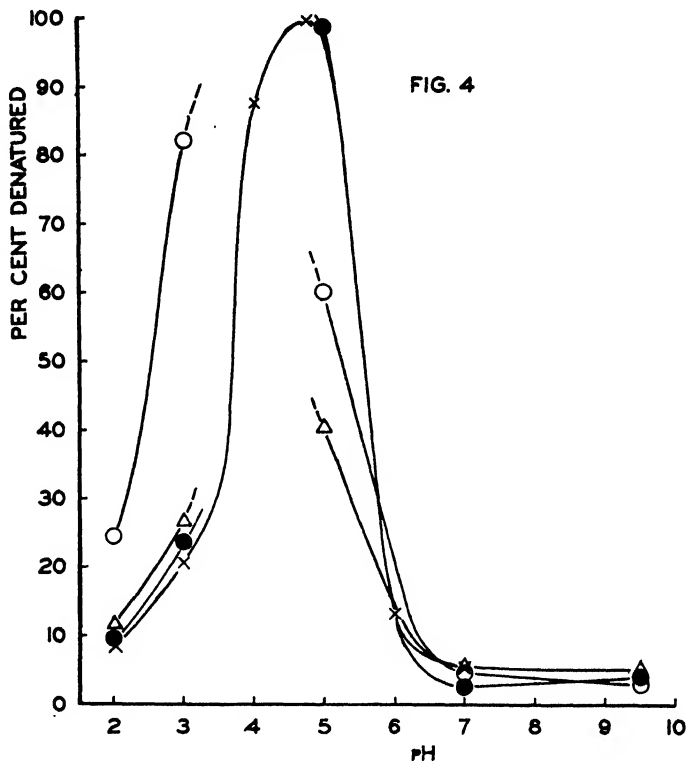
Fig. 4. Showing the extent of surface denaturation of a 1.05 per cent egg albumin solution after 18 hours shaking at several pH values with and without electrolyte. All solutions were brought back to the isoelectric point before analysis. ◯, addition of 0.01 N K_2SO_4; △, 0.01 N $BaCl_2$; ●, 0.01 N KCl; ✕, no electrolyte added.

gated at several pH values.   In these particular studies, after shaking for 12 hours in the 2° thermostat, the solutions were adjusted to the isoelectric point of the protein.   This adjustment

[1] Pauli and Weissbrod (3) found KCl, K_2SO_4, and also KCNS to favor the heat denaturation of ovalbumin and sucrose and to inhibit the process slightly.

always caused some additional precipitation of protein. The total amount of insoluble protein was taken as the amount actually denatured. Apparently in solutions somewhat away from the isoelectric point there is always a certain amount of denatured protein which remains in solution, owing probably to the electric charge which it possesses. The data are shown in Fig. 4. There is some evidence in the K_2SO_4 curve on the acid side (positively charged protein) for a valence effect. On the basic side there is either no effect or else the procedure was not delicate enough to measure it.
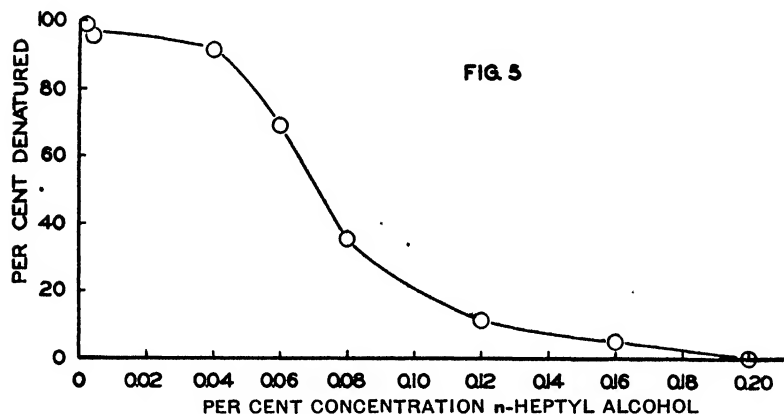
FIG. 5. Showing the inhibiting effect of *n*-heptyl alcohol on the rate of surface denaturation of a 1.05 per cent egg albumin solution; 12 hours shaking.

Sodium lauryl sulfate, to our surprise, promptly and completely coagulated the protein in as small a concentration as 0.0008 per cent. In concentrations higher than 0.008 per cent no precipitate was obtained but it was found that the pH had been shifted from the isoelectric point of the protein to pH 6.1, although the pH of the sodium lauryl sulfate was about 5. When the pH of the mixture was brought back to 4.8, complete precipitation of the denaturable protein resulted.

In order to study a surface-active material, the desired amount of a 1 per cent solution of *n*-heptyl alcohol in petroleum ether was added to the clean and dry 70 cc. bottles and the ether allowed to evaporate. 25 cc. of a 1.10 per cent protein solution were then

added and shaken for 12 hours.   The results are shown in Fig. 5. Even in concentrations as low as 0.04 per cent, the alcohol somewhat inhibited the denaturation and at 0.20 per cent there was complete inhibition.

<div style="text-align:center">DISCUSSION</div>

The process of surface coagulation seems to consist of at least two reactions: (a) The structure of the protein molecule is changed in some manner which results in a far more hydrophobic molecule than the natural protein.   Neurath (4) has described this as being an irreversible unfolding of the protein molecule at the surface.   Mirsky and Pauling (9) believe it to be associated with a loosening of the hydrogen bonds in the molecule; in any case, however, a relatively hydrophobic sol of denatured protein results.   (b) The second reaction consists in the precipitation or coagulation of the denatured protein sol.

As a first approximation, it is to be expected that the rate of surface denaturation would be governed by four reaction velocities: (1) the rate of diffusion of the protein molecules from the bulk of the solution to the surface; (2) the rate of spreading and denaturation of the protein on the surface; (3) the rate of formation of a new surface; (4) the rate of precipitation of denatured protein which, no doubt, takes place on the surface as well as in the bulk of the liquid.

Reaction (1) should be directly proportional to the protein concentration, and, if this were the controlling reaction, a first order reaction should result.   Accordingly when time is plotted against $\log (c')/(c' - x)$, a straight line should be obtained with the same reaction constant for all concentrations.   Although time against $\log (c')/(c' - x)$ does yield a fairly straight line, the velocity constant varies with the initial concentration, which should not be the case if diffusion were the governing factor (cf. Fig. 1).

The rate of spreading of the protein on the surface or how this is influenced by concentration is not known.   Neurath (4) reports that a protein film expands slowly to reach a maximum area in about 7 minutes.   The rate of spreading would be presumably inhibited by a higher concentration of protein at the surface, owing to a smaller area in which to spread, but on the other hand

there would be more molecules available for spreading, and it is difficult to say which would be the more important. There is no reason to believe that the ease of unfolding of the molecule, once it has reached the surface (spreading), should be decreased by positive or negative charges on the protein. On the contrary, it is conceivable that the electrostatic attraction between the zwitter ionic groups at the isoelectric point would increase the intramolecular cohesion forces, thus decreasing the ease of spreading rather than increasing it.

That reaction (3) largely influences the whole reaction is indicated by the following experiments. If the relative surface is increased by placing only 10 cc. of the solution in our 70 cc. bottles, an increased rate of surface coagulation should result. This was not the case if the normal rate of shaking was applied (96 movements per minute). On the contrary, as shown by Fig. 1, Curve 4, the coagulation velocity decreased, owing to the fact that this small quantity of liquid floated from one end of the bottle to the other without breaking of the surface and thus removing the coagulation products. If, however, the rate of shaking was increased to 140 movements per minute, a large increase of the coagulation velocity resulted (Fig. 1, Curve 5). *Vice versa*, a decrease of the relative surface by filling the bottle with 50 cc. of liquid produced a large decrease of the rate of coagulation, even when the higher rate of shaking was applied (Curve 6). When the bottle was filled completely, no surface coagulation took place. It is interesting to note that an increase of the rate of shaking has no influence on the reaction velocity when 25 cc. of the solution were used. Here, apparently, the lower rate of shaking is sufficient to remove the coagulation products and likewise to break the surface.

The protein, once it is denatured, should have only a very small tendency to leave the surface, since this represents a point of lower energy content. This is demonstrated experimentally by the fact that in a quiescent solution protein does not progressively denature on the surface. It is only by continually forming new surfaces by shaking that surface denaturation proceeds to any appreciable extent.

The rate of coagulation of the denatured protein may be a highly important factor in regions away from the isoelectric point,

since here we may have a considerable quantity of denatured protein still in solution, and there may be a competition for the available new surface between the natural undenatured protein and the denatured but still soluble protein.   The denatured but soluble proteins may act as an inhibitor in the same manner as does n-heptyl alcohol.   At first sight, the negative results obtained with gelatin might be regarded as opposed to this idea, but when it is remembered that gelatin is more hydrophilic than is natural egg albumin and certainly very much more hydrophilic than is denatured protein, it is not surprising that gelatin was without effect.   Its hydrophilic properties would tend to draw it out of the surface and into the solution.   Denaturation away from the isoelectric point does not proceed toward equilibrium but increases continuously with time, which is in keeping with the above suggestions.   In the stronger acid solutions, the denatured protein is again coagulated with a resulting increase in the rate of denaturation, due to a decreased activity of the protein ion beyond the pH range of maximal positive charge.

The above considerations probably explain Gorter and Philippi's (10) results of the influence of pH on the spreading of proteins. These authors obtained practically the same curve as that shown in Fig. 2 of this paper.   That is to say, the denatured protein being soluble away from the isoelectric point, some of it would go into solution and not remain on the surface.

Inasmuch as the change of the pH during shaking is an indication of the occurrence of denaturation, the results shown in Fig. 3 confirm our view that surface denaturation takes place throughout nearly the whole pH range.   The similarity between our curve and that of Pauli and Koelbl seems to indicate that the interaction between the protein molecule and the surrounding H ions of the solution is identical for both kinds of denaturation.   An explanation for this interaction must await further experiments.

As described in Paper I, no surface denaturation occurs when the protein has been previously denatured by heat.   We have again confirmed this point.   For example, 25 cc. of a 1.10 per cent solution were adjusted to pH 4.08 and heated at 100° for 15 minutes.   This water-clear solution was then shaken for 18 hours at the normal rate of shaking.   Not the slightest trace of surface coagulation was observed.   The same result was given by a protein

solution adjusted to pH 3.63.   These observations seem to indicate that by heat denaturation the intramolecular cohesion forces are strengthened to such an extent that the unfolding of the molecule at the surface is prevented.

SUMMARY

1. The dependence of the rate of surface denaturation on protein concentration has been studied, and it has been found that the higher the protein concentration (three concentrations of 2.10, 1.10, and 0.55 per cent) the lower is the rate of denaturation.

2. The extent of surface denaturation has been investigated as a function of pH for salt-free solutions, as well as solutions containing KCl, K_2SO_4, and $BaCl_2$.   Surface denaturation is greatly dependent on pH and proceeds fastest at the isoelectric point.

3. The pH has been found to change during surface denaturation, and this has been studied as a function of pH.

4. n-Heptyl alcohol was found to have marked inhibitory powers on surface denaturation.

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METABOLISM OF CARBOHYDRATE IN THE DEPAN-
CREATIZED DOG

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It has generally been accepted that there is no rise in respira-
tory quotient following the ingestion of carbohydrate by the
depancreatized animal and that the administered sugar is quanti-
tatively excreted.   However, careful scrutiny of the literature
reveals a surprising number of cases of apparent oxidation.
Falta, Grote, and Staehelin (13) reported no rise in R.Q. following
glucose ingestion, but they recovered only 65 per cent of the
administered sugar.   Verzár and von Fejér (27) presented a series
of ten experiments performed on the 4th to the 19th day after
pancreatectomy; of these, one dog had an average glucose R.Q.
of 0.78 after a basal of 0.71.   The remaining nine were negative
save for the fact that, in one other animal, a basal R.Q. of above
0.75 was obtained.   In this case glucose produced no rise.   In
another group of experiments reported by Verzár (26) no change
in quotient was found in five glucose tests after the 3rd postopera-
tive day.   On the 1st and 2nd days following extirpation of the
pancreas, elevated quotients were invariably obtained after glu-
cose injection.

Eight glucose experiments were performed by Moorhouse,
Patterson, and Stephenson (20) on· the 3rd to the 14th day after
operation.   One of these showed a rise from 0.74 to 0.77, while
six exhibited no change.   In the remaining one, glucose produced
an elevation in R.Q. from 0.73 to 0.80, but the next day, without
any glucose, quotients varied from 0.62 to 0.84.   Recoveries
ranged from 75 to 100 per cent, averaging 93 per cent, but the
low recoveries were not coincident with the heightened quotients.

Hédon (15) contributed a series of seven experiments in which

bread was fed to depancreatized dogs on the 3rd to the 21st day following removal of the pancreas explant.   Only one of these was entirely lacking in elevation of R.Q., while four showed unmistakable rises, often beyond 0.80.   Recently, Soskin (24) has disputed the earlier conclusions on the basis of the results of ten experiments, only three of which showed an appreciable rise in R.Q.   Glucose recovery averaged about 50 per cent for all the experiments, but this calculation was complicated by the effects of the feeding of meat twice daily.

The majority of quotients in the foregoing experiments remained unchanged following glucose ingestion, although in certain instances a rise was shown.   In contrast, two other types of experiments have indicated that the depancreatized preparation can oxidize carbohydrate, but in these there was some definite alteration purposely produced in the diabetic organism.   Basal quotients as high as 0.84 have been found by Hédon (15) and by Loubatières (18) in the "premortal" stages of fasting.   The usual rise in nitrogen excretion was noted but the amount was insufficient to account for the total metabolism.   No studies were made on the reaction of the animal to glucose administration in this condition.

Another factor has been introduced by experiments performed after removing the pituitary gland from depancreatized animals. In three of five depancreatized-hypophysectomized dogs Houssay and Biasotti (16) noted an elevation of the R.Q. from a basal level of 0.70 to approximately 0.80 after the administration *per os* of 50 gm. of glucose.   Similar results were obtained by Biasotti (3) on two more such animals after intravenous injection of the sugar.

In order to study the processes responsible for the contradictory results cited above, a careful survey should be made of carbohydrate metabolism in the simple depancreatized preparation. We are therefore presenting the evidence on the problem accumulated in this laboratory.

EXPERIMENTAL

These observations were made upon mongrel adult female dogs from which the pancreas was completely removed in a single stage operation and in which careful autopsy failed to reveal any

remaining pancreatic tissue. In general, urinary metabolism figures were obtained on the fasted animals daily, and the respiratory metabolism was determined for several hours before and after the administration *per os* of varying amounts of C.P. anhydrous *d*-glucose. Urinalyses were performed with the Van Slyke method for acetone bodies (25) and the Kjeldahl-Arnold-Gunning (21) and Benedict (2) techniques for nitrogen and for sugar, respectively. Preformed creatinine was determined by the procedure proposed by Blau (4); the amount of creatine was obtained by subtracting this figure from the value for the "total creatinine" obtained by the Folin technique (14). These two methods were found to be the most suitable ones for urines containing both acetone bodies and glucose. For the sake of convenience, the experiments are divided into two series, according to the type of respiration apparatus used.

.*Series 1. Calorimeter Experiments*—The data of this series include the respiratory metabolism determinations which were made in the Lusk calorimeter and the chemical analyses of urinary glucose and nitrogen from the studies of several investigators in this laboratory during the past 10 years. We are greatly indebted to Doctors M. A. Kennard, E. Marquis, and A. C. Santy for their aid in these experiments and the use of their data.

The accuracy of the calorimeter has been established during the time of these experiments by the alcohol checks published in the "Animal calorimetry" series. For brevity only a summary of the respiratory data is given in Table I. The dogs were fasted for 24 or 48 hours before pancreatectomy and thereafter until the glucose was given, except in the two instances noted in the table. In most cases a glucose experiment was conducted immediately after a determination of the basal metabolism. The figures given in Table I for basal metabolism represent the average of two to four consecutive hourly periods, and for the glucose experiments the average of the 2nd, 3rd, and 4th hours after ingestion unless a second dose of sugar was administered at the end of the 3rd hour.

The experiments are arranged in Table I according to the length of fast from pancreatectomy until the glucose was given. The basal R.Q.'s ranged between 0.70 and 0.73, which is in agreement with the results of the earlier workers tabulated by Rapport

((22) p. 432).   The amount of glucose ingested varied from 16 to 50 gm.   In four experiments the technique was followed by means of which Wierzuchowski (28) was able to demonstrate the oxidation of glucose in the phlorhizinized dog; *i.e.*, a second 25 gm. dose was given 3 hours after the first one.   In the two ex-

TABLE I

Effect of Ingested Glucose on Respiratory Metabolism of Fasted Depancreatized Dogs

Dog No.	Days post-opera-tive	Basal metabolism			Glu-cose per os	Glucose metabolism				
		R.Q.	Calcu-lated heat	Direct heat		R.Q.	Calcu-lated heat	Direct heat	Specific dy-namic action	Quiet hrs.
			calories per hr.	*calories per hr.*	*gm.*		*calories per hr.*	*calories per hr.*	*calories per hr.*	
87	4	0.70	26.03	26.44	45	0.71	30 69	31.59	·	None
98	5	0.73	16.56	17.32	25	0.71	18.16	18.31		"
					25	0.69	18.55	17.91	2.0	1
82	6	0.71	19.84	18 32	45	0.72	20.39	19.28		None
91	6	0.72	23 43	23.99	25	0.71	24.36	25.93	0 9	2
					25	0.71	30.27	30.33		None
101	6	0.72	16.95	14.27	25	0.67	21.68	18.42		"
	7				25	0.70	20.08	16.87		"
					25	0.73	19.69	21.89		"
90	6	0.71	20.75	23.31	50	0.75	21.77	23.81	1.0	1
	7				25	0.74	21.17	25.02	1.0	2
	8	0.72	19.63	21.75						
78	11	0.73	21.32	22.39	16	0.72	21.74	22.29	0.4	2
79	10	0.72	24.07	19.18	16	0.73	24.63	22.99		None
	13*	0.73	22.61	18.91	16	0.73	23.40	20.94		"
	17	0.72	19.47	16.28	16	0.71	20.93	18.10		"
98	5†	0.71	14.67	15.55	25	0.71	16.04	14.39	1.4	1
					25	0.71	17.49	18.83		None
140	5‡	0.73	27.07	26.69	50	0.73	29.81	29.83		"

* 100 gm. of meat on the 12th day.

† 5 days after insulin; 200 gm. of meat daily.

‡ 5 days after insulin.

periments on Dog 90 the validity of a rise is doubtful, since the average quotients of 0.75 and 0.74 are in each case due to a single high R.Q. of 0.76 in only 1 out of 4 hours.   The animal was exceptional in that enlarged and tortuous mesenteric veins anastomosing with the splenic vein were noted at the time the pan-

creas was removed. This condition was explained by the autopsy finding of a complete obstruction of the portal vein just below the entrance of the splenic.

In the past, little attention has been paid to the specific dynamic action of glucose in the depancreatized animal. Table I contains the figures for the heat production, expressed in calories per hour, and calculated from the oxygen consumption and the non-protein R.Q. in the conventional manner. In all of the experiments the calculated heat production after the ingestion of sugar was higher than that of the basal periods, but the quantitative expression of the relation is of little value in some of the experiments because of movement. The six cases in which quiet periods were obtained show an average of about 1 calorie per hour of extra heat above the basal level, as calculated from the oxygen used. When movement occurs, direct measurement of the heat output is of distinct advantage, since the temperature changes are read every 4 minutes and the heat production for the quiescent part of the hourly period can be estimated. These estimations, although not included in the data, furnish corroborative evidence of a definite specific dynamic action.

The calculation of glucose recovery from the amount of glucose excreted depends upon a constant glycogen content of the body and a constant production of sugar from protein. The latter is indicated by the basal urinary D:N ratio. Such a condition is maintained in the phlorhizinized animal by frequent injections of the drug. Macleod (19) contended that the D:N ratio was constantly falling after pancreatectomy. The daily basal ratios of all the fasted depancreatized dogs used in this laboratory for exercise, glucose, or other experiments have been assembled and are grouped according to the length of fast prior to pancreatectomy. The averages for the groups are given in Table II. A preliminary fast of 2 or 3 days apparently reduces the glycogen stores sufficiently to give a fairly uniform D:N ratio at about the Minkowski level of 2.8 for 5 to 7 days. Longer postoperative periods of fasting produced more irregular and occasional low ratios, such as 1.91 and later 2.96 in Dog 79 (Table III), and in Dog B-18 (Table V) 0 on the 12th day and 3.02 on the 19th day. Despite a few such fluctuations, the general constancy of the D:N in the fasting depancreatized animal would indicate that

the use of such a ratio in calculating "extra glucose" recovery is the most reliable procedure thus far suggested.

Since the daily D:N ratios of two of these animals and of a number of others have been published previously (9), and all are included in Table II, only the individual ratios which were used in the calculation of the recovery of extra glucose are shown in Table III. The first column of basal D:N ratios ("Before") are the figures for the 24 hour period preceding the glucose experiments. Extra sugar was calculated from the nitrogen and glucose excreted in the 24 hours after ingestion. In some instances the urine of the succeeding day, when the animal had returned to a basal level, was obtained ("After," Table III), and in these cases the average of the two basal ratios was used to calculate the

TABLE II

Average 24 Hour D:N Ratios

No. of dogs	Pre-operative fast	Postoperative days					
		2	3	4	5	6	7
	hrs.						
10	24	3.40	3.22	3.07	3.10	3.08	2.95
20	48	3.05	2.93	2.82	2.84	2.53	2.61
5	72	2.77	2.86	2.89*	2.94		

* One ratio of 4.70 omitted from the average.

glucose derived from endogenous protein. The percentage of administered glucose which was recovered in the urine is shown in Table III. In the five experiments in which 16 or 25 gm. were given, from 86 to 104 per cent, or an average of 97 per cent, was excreted. With the larger amounts, 45 or 50 gm., in single or in divided doses, the eight experiments showed a yield of from 77 to 93 per cent, with an average of 88 per cent.

Series 2. Experiments with Open Circuit Apparatus—An open circuit type of apparatus was used in obtaining the respiratory data reported in this section, the air analyses being done by use of the Carpenter-Haldane gas analyzer (6). Samples of the air leaving the chamber were drawn continuously by means of a mechanical device, thus insuring a representative aliquot for the entire duration of each hourly respiration period. The out-

going air was passed through three Williams bottles (29) containing a bisulfite solution to absorb acetone, which was then determined as previously outlined (1).   These bubblers also served to saturate the air with moisture in order that the ventilation could be measured by means of a wet-meter.   The accuracy of the method was tested at intervals throughout the course of the

TABLE III

Glucose Recovery and Nitrogen Excretion

Dog No.	Days of fast	Body weight		Glucose		Basal D:N		Nitrogen excretion per hr.			
								Basal, 24 hrs.	After glucose		
		Pre-operative	Loss	Fed	Recovered	Before	After		Metabolism period	24 hrs.	
		kg.	*per cent*	*gm.*	*per cent*			*mg.*	*mg.*	*mg.*	*per cent*
87	4	12.5	9	45	92	2.76		259	271	197	76
98	5	8.4	17	25		2.43		213	262		
				25	85		2.64		208	197	92
82	6	8.7	16	45	90	2.35		163	143	81	50
91	6	10.5	18	25		2.75		404	366		
				25	92		2.55		365	349	86
101	6	8.5	20	25	104	2.52		187	244	226	121
	7			25					223		
				25	77				270	223	119
90	6	12.5	13	50	92	3.30		269	304	264	98
	7			25	86	2.82		217	230	217	100
78	11	13.0	22	16	103	3.13	3.10	256	300	221	86
79	10	15.6	21	16	92	1.91	2.23	255	355	260	102
	13*		25	16		2.77		247	351		
	17		33	16	101	2.96	2.01	392	556	559	143
140	5†	13.7	12	50	93	2.49		277	418	256	92

* 100 gm. of meat on the 12th day.

† 5 days after insulin.

work by means of the usual alcohol and acetone checks.   Seventeen alcohol R.Q.'s, each figure representing at least three 1 hour periods, varied between 0.665 and 0.673, with an average of 0.669 ± 0.004.   Five acetone quotients yielded an average of 0.751 ± 0.002.

All of the dogs reported in this section were maintained for 3

TABLE IV

Effect of Ingested Glucose on Respiratory Metabolism of Fasted Depancreatized Dogs

Dog No.	Days after insulin	Body weight		Basal metabolism		Glucose		Glucose metabolism		
		Pre-operative	Loss	R.Q.	Calculated heat	Fed	Recovered	R.Q.	Calculated heat	Specific dynamic action
		kg.	*per cent*		*calories per hr.*	*gm.*	*per cent*		*calories per hr.*	*calories per hr.*
B- 3	4	11.8	16	0.72	23.45	25		0.74	23.09	0
						25*	96	0.73	23.22	0
B-16	4	15.3	35	0.73	20.68	25		0.73	23.93 (*m*)	
						25*	104	0.73	26.01 "	
B-20	4	11.3	38	0.71	16.83 (*s*)	25		0.71	16.80 (*s*)	0
	.					25*	104	0.73	16.69 "	0
B-17	5	10.8	39	0.73	14.47	25		0.72	14.98	0.5
						25*	99	0.71	14.52	0
B-18	5	15.4	35	0.72	24.33 (*s*)	25		0.72	25.14 (*s*)	0.8
						25*	94	0.73	25.24 "	0.9
B-11	6	12.3	23	0.71	17.12	25		0.71	17.20	0
						25*	98	0.71	12.27 (*s*)	0
B-13	6	12.7	15	0.69	26.04	25		0.71	26.99 "	0.9
	.					25*	98	0.72	27.78 (*m*)	
B-18	12	15.4	44	0.72	18.90	25		0.72	21.31 "	
						†	92	0.71	18.94 (*s*)	0
B-18	19	15.4	50	0.71	18.51	25		0.72	18.87 "	0 3
						†	99	0.73	18.68	0
Premortal										
B-17	16	10.8	49	0.81	8.59	25		0.88	9.95	1.4
						25*	29	0.90	11.99	3 4
B-18	22	15.4	55	0.72	17.47	25		0.77	13.97	
						†	40	0.78	13.39	
B- 5	23	10.3	55	0.72	11.61	25		0.77	10.32	
						†	45	0.78	13.02	

m, indicates that movement invalidated heat production of all hours; *s*, quiet hours were selected for calculation of heat production.

* A second dose of glucose was administered at the end of the first respiration period.

† The animal was replaced in the chamber for a second metabolism period; no second dose of glucose.

or 4 days after the pancreatectomy[1] on food and insulin.[2]   Then a period of at least 3 days without food or insulin was imposed before any experiments were conducted.   In Table IV each experiment shown consists of a basal metabolism period extending over 3 to 4 hours, and two similar periods following 25 gm. of glucose administered *per os*.   In most experiments a second 25 gm. dose was given following the expiration of the first 4 hours.   Table IV lists the respiratory data of these experiments in order of length of fast following withdrawal of food and insulin. The first nine experiments, performed on the 4th to the 19th day after insulin injection, show only one glucose R.Q. as high as 0.74 and no instance in which the quotient rose significantly above the basal.   This condition was fulfilled even though one dog had lost as much as 50 per cent of its original body weight. Striking contrasts are shown by the last three experiments, in each of which the administration of glucose caused an unmistakable rise in R.Q.   This elevation of quotient is the more remarkable because in the cases of Dogs B-5 and B-18 there was no evidence of the typical premortal rise, either in basal R.Q. or in nitrogen excretion (see Table V).   The second of these two animals is particularly interesting when it is noted that the reaction of this dog (No. B-18) to glucose was tested on the 5th, 12th, and 19th days following withdrawal of food and insulin.   In all three instances the customary lack of response was shown, yet on the 22nd day of fast a marked rise in R.Q. was obtained.

Since calorimetric observations of direct heat production were not made in· this series, the activity records of the animals furnished the sole criteria for accepting or discarding the indirect heat·for each hour as computed from the oxygen consumption in the customary manner for diabetic animals.   Table IV shows the caloric outputs marked *s* when certain hours were selected because of lack of movement, or marked *m* when movement invalidated all 3 hours.   When no notation is shown, the animal was uniformly quiet.   Judged on this basis, there were three rises· of 0.8 to 0.9 calorie each, the remainder being only insignificant changes.

The amount of extra glucose excreted by each dog was calculated in the manner outlined before. The average return of the first nine experiments was 98 per cent, with the individual amounts ranging from 92 to 104 per cent (Table IV). Even the

TABLE V

Effect of Ingested Glucose on Urinary Constituents during Metabolism Periods

Dog No.	Days after insulin	Basal				Glucose		
		Acetone bodies		Urine N	D:N	Acetone bodies		Urine N
		Total	Urine			Total	Urine	
		mg. per hr.	*mg. per hr.*	*mg. per hr.*		*mg. per hr.*	*mg. per hr.*	*mg. per hr.*
B- 3	4	155	142	199	2.49	130	116	203
						121	103	162
B-16	4	57	46	136	2.88	64	48	190
						85	60	196
B-20	4	84	78	137	4.29	80	71	137
						87	76	143
B-17	5	15	13	124	2.90	17	10	125
						14	11	120
B-18	5	19	18	124	2.83	24	23	137
						25	25	148
B-11	6	14	14	96	1.88	24	21	127
						34	30	135
B-13	6	157	136	288	2.80	122	95	330
						117	84	308
B-18	12	14	11	110	0	23	20	145
						26	25	178
B-18	19	16	12	158	3.02	14	13	186
						16	14	191
Premortal								
B-17	16	0	0	305	0	0	0	186
						0	0	237
B-18	22	25	24	117	0.95	14	13	144
						11	11	132
B- 5	23	30	28	95	2.36	8	6	80
						12	9	82

second test on Dog B-18, starting with the basal urine sugar-free, did not fall below the "quantitative recovery" range of 90 to 110 per cent. By way of contrast, the recoveries from the three premortal dogs were extremely low. Two doses of 25 gm. each were given to Dog B-17 and only about 14 gm. were recovered.

The other two animals received only 25 gm. apiece and returned 40 and 45 per cent, respectively.

In Table V are listed the changes in total acetone and in urinary acetone and nitrogen during the periods the animals actually were in the respiration chamber. Although many of the changes are of doubtful significance, owing to the glucose polyuria, the values are given in order to make possible a comparison with the corresponding values for phlorhizinized dogs (28). In order to

TABLE VI

Effect of Glucose Ingestion on Urinary Constituents (in Mg. per Hour) during Prolonged Periods

Dog No.	Days after insulin	Basal						Glucose					
		Hrs.	Acetone bodies		Urine N	Creatinine	Creatine	Hrs.	Acetone bodies		Urine N	Creatinine	Creatine
			Total	Urine					Total	Urine			
B- 3	4	16.69	147	134	191			20.26	149	134	164		
B-16	4	12.24	49	37	143	9.0	2.5	20.91	76	61	172	14.5	3.0
B-20	4	3.72	84	78	137	8.5	3.5	19.84	107	100	153	10.3	5.0
B-17	5	13.72	12	10	130	11.0	8.5	15.63	16	12	128	9.5	10.5
B-18	5	19.83	23	22	129	10.0	0.5	21.59	24	23	133	10.5	1.0
B-11	6	14.67	12	11	91	11.5	1.0	21.21	25	22	98	12.5	1.0
B-13	6	19.12	151	133	262	15.0	9.0	20.94	129	104	253	17.0	14.5
B-18	12	30.60	23	21	153	8.5	8.0	20.72	21	20	121	8.5	2.5
B-18	19	28.98	17	14	166	8.0	10.0	18.35	18	16	196	9.5	7.5
Premortal													
B-17	16	20.21	0	0	314	4.5	18.5	20.21	0	0	182	7.0	18.5
B-18	22	15.42	26	25	120	8.5	21.5	15.42	12	11	126	5.5	16.0
B- 5	23	13.11	35	33	103	4.0	19.0	13.11	12	10	87	4.5	16.0

avoid misinterpretation of changes in metabolism as measured by urinary excreta during diuresis, the values obtained for the respiration periods have been combined with other preceding or subsequent periods. Table VI contains averages for basal and for glucose periods extending over 15 or more hours. The figures given for total acetone bodies are made up of urine data plus calculated values for expired air acetone. The latter were obtained by actual determination of the amount produced during the respiration periods added to computations at the basal rate

for the remaining time.   Creatinine and creatine values also are
included in Table VI.

The commonly accepted criteria for oxidation of ingested carbo-
hydrate are an elevation in R.Q., a corresponding diminution in the
amount of extra glucose excreted, a protein-sparing action, and a
ketolytic effect.   Wierzuchowski clearly demonstrated that all
these were satisfied in the phlorhizin-treated dog after the inges-
tion of glucose, being especially marked following the administra-
tion of two doses of glucose separated by 3 hours.   In one typical
experiment, from a basal level of 0.70, the R.Q. rose to 0.80 for
the 1st hour after the second 20 gm. dose.   During successive
hours the quotients were 0.74, 0.77, and 0.71.   The recovery of
extra glucose was only about 80 per cent.   Following ingestion
of 29 gm. of sugar by another phlorhizinized dog, the nitrogen
excretion decreased 12 per cent in the first 3 hours, 39 per cent
in the second 3 hours, and 46 per cent in the third.   During the
same periods the ketonuria fell 59, 90, and 87 per cent, respec-
tively.   In this instance recovery of extra sugar was 71 per cent.
These observations have since been confirmed by Deuel, Wilson,
and Milhorat (12) and by Boothby, Wilhelmj, and Wilson (5),
who also could recover only about 80 to 90 per cent of the ingested
sugar from the urine.

Our data presented above indicate two definite stages in the
history of the fasting depancreatized dog, judging from the reac-
tion of the animal to ingested carbohydrate.   The first phase
was from 4 days after the removal of the pancreas or the cessa-
tion of insulin injection until about 45 to 50 per cent of the body
weight had been lost, during which period oxidation of ingested
carbohydrate did not appear to take place.   All of the animals
listed in Tables I and III and the first nine in Tables IV, V, and
VI are considered as being in this category.   When the weight
loss reached about 50 per cent the animal passed into the second,
or premortal stage, in which the metabolism was definitely
changed.   These two conditions will be treated separately in the
ensuing discussion.

Examining the data gathered on the dogs in the first stage, we
find that in no case was there a definite rise in R.Q. over the basal

level following administration of glucose. In a few instances the quotient increased slightly, but the largest of these changes was only 0.04, at best an increment of doubtful significance. The average of all post-glucose quotients is found to be 0.72, exactly the same figure obtained as an average of all the basal periods. Since we are reporting the results of twenty-two experiments, using two different means of studying the respiratory metabolism, such a lack of change in R.Q. does not seem to be a matter of coincidence.

The glucose experiments on depancreatized animals reported prior to the discovery of insulin in 1922 were performed during a period starting immediately after the operation. Series 1 of our experiments (except on Dog 140) was conducted in that manner, but in Series 2 we followed the newer technique of maintaining the animals for a few days after the pancreatectomy on a liberal diet with sufficient insulin to keep the urine nearly sugar-free. It is' interesting to note that the results produced by two such different methods of preparing the experimental animals are, in general, identical. On Dog 98 (Table I) both procedures were used, the first glucose experiment being performed on the 5th day of fast after pancreatectomy. Food and insulin were then given for 8 days; following this the insulin was withheld, but 200 gm. of lean beef heart were fed daily for 4 days. On the 5th day a second glucose experiment gave the same negative results as the first.

An interesting observation is that the basal quotients did not progressively rise as the fasting state was prolonged beyond 5 days. Hédon presented data which he interpreted as showing that, beginning with the 3rd day after complete pancreatectomy, the R.Q. slowly rose from 0.72 to reach 0.75, and often 0.76, by the 8th day (15). On the contrary, in both of our series of animals the basal quotients were not above 0.73, despite the fact (Tables I and IV) that many were obtained on the 6th, 7th, and 8th days of fast. Furthermore, in the case of Dog 79 on the 10th, 13th, and 17th days postoperative and Dog B-18 on the 5th, 12th, 19th, and 22nd days after withdrawal of food and insulin, basal R.Q.'s varied from 0.71 to 0.73 without showing any ascending sequence.

A temporarily increased nitrogen excretion in most of the ex-

periments accompanied the polyuria brought on by the glucose administration (Table III, metabolism period, and Table V). This fact becomes especially significant when it is remembered that the phlorhizinized dog exhibited a prompt and undoubted fall in urine nitrogen, despite the development of the usual polyuria following glucose ingestion. In order correctly to assay changes in protein metabolism as evidenced by nitrogen output during the diuresis, the figures obtained for the shorter periods were combined with those of the overnight urines to yield basal and experimental nitrogen values for 15 or more hours. When these figures are examined (Tables III and VI), it is seen that there was no significant change whatever in nearly half the instances. The remainder were so evenly divided between a rise and a fall that it appears doubtful from these data that any protein-sparing action was exerted by the glucose.

Chaikoff and Weber (7) and Selle (23) have previously presented data indicating that there is no alleviation in the ketosis of depancreatized dogs following injection or ingestion of glucose. Other data are less conclusive (20, 24). The open circuit series of our experiments added the advantage of enabling the quantitative collection of acetone in the expired air, thus making the collection of the ketone body excretion complete. It is found that only two animals (Dogs B-3 and B-13, Table V) showed even a temporary acetone-sparing effect. When the results for the longer periods are examined (Table VI), no change is revealed in most of the experiments, and a definite ketolysis only in the case of Dog B-13. Whatever may be the explanation for the instances of increased nitrogen and acetone excretion, certainly the invariable pronounced fall in excretion of both substances produced by ingested carbohydrate in the phlorhizinized animal is completely lacking in these experiments.

Clark and Murlin (10) have recently claimed a ketolytic and nitrogen-sparing action for glucose in the depancreatized dog, with no accompanying rise in R.Q. In their experiments the dogs were always fed meat on the night preceding a test, but no control experiments were included on a possible subnormal rate of digestion and absorption of protein after pancreatectomy. From examination of the data presented, it appears as though the nitrogen-sparing action and the ketolytic effect might be ques-

tioned on the basis that the heightened output of these substances following meat ingestion was not complete at the time the "basal" urines were obtained.   Thus, the true basal postabsorptive nitrogen and acetone levels may not have been reached until the periods following sugar administration.

The average recovery of glucose in the urine for the twenty-one experiments of both series was 95 per cent.   A possible explanation for the lower recovery values obtained in some of the experiments is suggested by the specific dynamic action data.   The figures in Table I show increases in heat production of about 1 calorie per hour when the animals were quiet, and those in Table IV demonstrate a rise of 0.5 to 0.9 calorie in four of fourteen experiments.   Although a rise of 1 calorie is small, no significant decrease occurred in any of the experiments.   This is in contrast to a fall of about 10 per cent in the phlorhizinized dog when approximately 20 per cent of the sugar was oxidized (5).   Evidence from the fasted dog indicates that the specific dynamic action of glucose may be associated with glycogen formation rather than with the oxidation of the carbohydrate (11).   In the depancreatized dog a temporary retention of glucose as glycogen might retard its rate of excretion and reduce the amount recovered in a 24 hour period.   Several of our experiments showing an increased heat production have a glucose recovery below the average but the data are inadequate for any definite correlation.

We have remarked that, taken separately, the criteria of carbohydrate oxidation are satisfied in such a small number of instances as to suggest the complete loss of oxidation.   It is of additional interest to note any coincident changes which may have occurred in certain animals, indicating tendencies different from the group as a whole.   Even though rather low glucose recoveries were noted in certain of the calorimeter series, there was found no accompanying change in R.Q or nitrogen excretion.   In the second series, introduction of the acetone values increased the possibility of coincidences, but examination of the data reveals that in no case did more than two of the constituents studied change together.   The two instances of nitrogen-sparing action (Dogs B-3 and B-18, Table VI) exhibited no changes in R.Q or acetone excretion, although in one the recovery was slightly lower than the average.   The one instance of ketolytic action,

Dog B-13, was accompanied by no change in the other factors. Thus, the few random changes produced in the metabolism of depancreatized dogs by the administration of glucose present a picture totally different from the evident oxidation of glucose in the case of the phlorhizinized dog.

Entirely at variance with the lack of response to carbohydrate shown in the first stage of fasting is the change found in the three animals termed premortal. Even though only one (Dog B-17) showed the elevation in basal R.Q. and protein breakdown pointed out in the depancreatized dog by Hédon, all three exhibited signs of a carbohydrate metabolism unmistakably altered from the usual diabetic response. Dog B-5 reacted to administered glucose with both ketolytic and nitrogen-sparing responses, while Dog B-18 had a definite ketolytic but no nitrogen-sparing action. Both excreted less than half the usual amount of extra glucose. The average of the heat production of the two metabolism periods on Dog B-5 was the same as the basal level, but in Dog B-18 it fell 3.5 calories during the first period and 4.1 during the second.

The typical premortal syndrome was shown by Dog B-17, and this animal responded to glucose with a rise in R.Q. and heat production, as well as with a fall in the heightened protein breakdown. There was no acetone present from the start, as might have been expected from a basal R.Q. of 0.81. The absence of glycosuria was confirmed by a blood sugar of 37 mg. per cent; this low value stands in contrast to the diabetic levels of 381 and 259 mg. per cent existing in Dogs B-5 and B-18, respectively, during the basal periods of their premortal experiments.

No preliminary glucose experiment was performed on Dog B-5, but in an earlier test the ingestion of 350 gm. of meat did not produce any rise in R.Q. above the basal quotient of 0.71, and the D:N ratio remained at the diabetic level of 2.80. It is therefore justifiable to conclude that the terminal condition of this animal in some manner changed its reaction to carbohydrate. The other two dogs had earlier control experiments (Tables IV to VI) which showed a complete lack of oxidation of the ingested carbohydrate. It is especially worthy of attention that three preliminary glucose tests were conducted on Dog B-18, the last one being completely

negative, even though only 3 days before the premortal condition was revealed.

Howe and Hawk (17) first called attention to a rise in creatinuria concurrent with a breakdown of body protein when the normal dog was subjected to prolonged fasting. In our experiments this correlation is evident only in the case of Dog B-17. On the 5th day after food and insulin this animal was excreting 11 mg. per hour of creatinine and 8.5 mg. per hour of creatine (Table VII). When the animal exhibited the typical premortal

TABLE VII

Creatinine and Creatine Excretion of Long Fasted Depancreatized Dogs

Dog No.		Days after insulin											
		5	6	10	12	14	15	16	19	20	21	22	23
B-17	Weight loss, %	39	41	43	44	45	47	49					
	Creatinine*	11.0	10.0	7.0	6.5	6.0	6.0	4.5					
	Creatine*	8.5	10.5	8.0	8.0	11.5	15.5	18.5					
B-18	Weight loss, %	35	36		43	44	46		50	51	52	55	
	Creatinine*	10.0	9.5		8.5	7.5	9.5		8.0	8.0	11.5	8.5	
	Creatine*	0.5	1.0		8.0	2.5	5.0		10.0	13.0	21.0	21.5	
B- 5	Weight loss, %	24		37	41	44	46		50	51	52	54	55
	Creatinine*	10.5		10.0	9.0	9.5	8.5		6.0	6.0	5.5	5.0	4.0
	Creatine*	6.5		7.5	8.0	9.0	8.0		7.0	9.5	12.0	18.5	19.0

* The figures for creatinine and creatine are expressed as mg. per hour over approximately 15 hour periods.

syndrome 11 days later, the creatinine output had fallen to 4.5 mg., while the creatinuria had more than doubled, to reach an hourly level of 18.5 mg. The creatinine and creatine excretions of Dog B-18 were evenly balanced through the period during which the first three glucose experiments were performed. These tests all showed no evidence of oxidation of the sugar. After the third experiment the creatine excretion suddenly rose until it was at an even higher level than that of Dog B-17. When glucose was given to the animal in this condition an appreciable portion apparently was oxidized. The same terminal rise in

creatinuria was shown by Dog B-5, which also exhibited a capacity to burn the administered carbohydrate, although no rise in basal R.Q. had occurred. None of the data on animals still refractory to glucose showed such a preponderance of creatine over creatinine (Table VI).

These results are quite similar to data acquired in this laboratory on the fasted dog with intact pancreas (8). After periods of 2 to 4 weeks without food a depression in oxidation of carbohydrate was produced. As the fast was continued until the weight loss was about 50 per cent, the creatine excretion rose, and the ingestion of glucose again induced an early rise in R.Q. Furthermore, all of the animals did not exhibit the typical premortal elevation in basal R.Q. and in endogenous protein metabolism.

The current explanation of the rise in creatinuria during prolonged fasting is that the substance comes from the greatly increased breakdown of actively functioning body tissue. Our observations on animals approaching the premortal state indicate a definite change in creatine excretion and in carbohydrate metabolism without always involving the total nitrogen output. Whether or not the response to glucose, although strongly suggestive, actually does signify carbohydrate oxidation cannot be settled from the data at present available. It might be considered that these experiments invalidate the earlier ones which show a lack of oxidation, but we do not believe this to be the case. A sudden, marked alteration in the functioning of the organism was produced as it passed from the stage of non-oxidation of glucose to that of apparent oxidation. The only clue to the change thus far discovered is the rise in creatinuria, and the significance of this finding is not yet known.

SUMMARY

In the early and intermediate stages of inanition the depancreatized dog exhibited no rise in R.Q., no nitrogen-sparing effect, and no ketolytic action following the ingestion of 16 to 50 gm. of glucose, in single or in divided doses. The extra glucose recovered in the urine averaged 95 per cent for twenty-one experiments. These results indicate no oxidation of the administered sugar.

As the animal approached the last stages of inanition, in which the creatinuria increased markedly, the typical effects of carbo-

hydrate oxidation were obtained, regardless of whether or not the Hédon premortal syndrome was found.

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THE EXCHANGE OF SALT AND WATER BETWEEN MUSCLE AND BLOOD

II. THE EFFECT OF RESPIRATORY ALKALOSIS AND ACIDOSIS INDUCED BY OVERBREATHING AND REBREATHING

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WITH THE TECHNICAL ASSISTANCE OF NATALIA TUPIKOVA

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In Paper I of this series (1) the effect of the injection of isotonic salt solutions on the extra- and intracellular phases of muscle was considered. In addition to the observed increase in the extracellular phase in these experiments, it was also found that the injection of both alkalinizing and acidifying solutions resulted in an increase in the intracellular phase.

The purpose of the present paper is to present data on the changes produced in the volume phases of muscle when the pH of the blood is changed simply by varying its carbon dioxide tension *in vivo*. Specifically, respiratory alkalosis was induced by forced overventilation and respiratory acidosis by continued rebreathing in a closed system in the presence of an excess of oxygen.

Since 1897, when Loeb (2) observed that the muscles of frogs swell more in alkaline solutions than in acid solutions of the same concentration, there have been numerous investigations along similar lines, which in general confirm this observation.

However, experimental data do not seem to be available for evaluating the effect produced on the extra- and intracellular phases of mammalian skeletal muscle by altering the carbon dioxide tension of the blood *in vivo*. The recent work of Fenn and Cobb (3) deals with an *in vitro* study of frog muscle and blood maintained at different carbon dioxide tensions. Since potassium determinations in their experiments indicated that the muscles had retained their normal permeability relationships, it

may be assumed that their results approximate those which one might find *in vivo*.

Fenn and Cobb (3) studied the chloride and water of frog muscle after immersion in frog blood maintained at carbon dioxide pressures corresponding to 7 and 70 mm. respectively. From these data they calculated the extracellular phase (called by them the "chloride space"). The difference between the chloride space at high and low carbon dioxide tensions was sometimes positive and sometimes negative, although the average of nine experiments indicated that the extracellular phase was 24 gm. per kilo of muscle greater at the higher carbon dioxide pressure. Owing to the irregularity of the results, no significance was attached to the difference by the authors. However, it may be pointed out that, since there was no significant difference in the average weight changes of the muscles, the average increase in the extracellular phase, if real, must have been at the expense of the intracellular phase.

<div align="center">EXPERIMENTAL</div>

Physiological Procedures

Normal healthy dogs (used in all experiments) were anesthetized by sodium barbital given intraperitoneally in doses of 270 mg. dissolved in 2.7 cc. of water per kilo of body weight. The blood pressure was continuously recorded from the carotid artery.

Overbreathing Series—After cannulation of the carotid artery and dissection of the trachea, 25 to 30 cc. of blood were taken under oil from the femoral artery, and one of the rectus abdominis muscles was removed, for the control chemical analyses. The trachea was then cannulated and connected by a short rubber tubing to two Palmer respiration pumps (4) which were operated synchronously at 160 respirations per minute. Hyperventilation was induced by equal adjustment of the positive and negative pressures and was continued for 1 hour. The second sample of blood was then taken from the femoral artery under oil, and the opposite rectus abdominis muscle removed, for the final analyses.

Rebreathing Series—After the carotid artery was connected with the mercury manometer, 30 cc. of blood were taken from the femoral artery under oil, and one of the rectus abdominis muscles was removed, for the control analyses. The animal was then

connected by means of a mask to a Krogh respiration apparatus whose bell was filled with oxygen.   The outlet valve to the apparatus was closed to allow the accumulation of carbon dioxide, after which oxygen was introduced at intervals throughout the experiment to prevent oxygen lack.   The animal breathed into and rebreathed from the spirometer for 60 minutes, after which the rectus abdominis muscle on the opposite side was removed and blood again taken from the femoral artery under oil for the final analyses.

The following determinations were made on the serum: pH, carbon dioxide, water, chloride, protein, sodium, and total base; on the muscle: water, chloride, sodium, and total neutral fat. Muscle and blood were removed and treated in the same way and with the same uniformity of technique as in the preceding experiments and the chemical methods were the same in detail as those previously described (1).   The analytical results on muscle are reported in terms of fat-free tissue.   The carbon dioxide tension of the blood, pCO_2, and the bicarbonate of the serum, $(BHCO_3)_s$, were calculated from the carbon dioxide content and the pH values with the Henderson-Hasselbalch equation and the values 6.10 for pK'_s and 0.510 for αCO_2.

Results

Ten experiments were performed, including five alkalosis experiments and five acidosis experiments.   In Tables I and II are given the data of each group.   Those not included did not differ essentially from those reported.

Effects of Respiratory Alkalosis (Table I)—During the hyperventilation, the blood pressures decreased 8, 32, and 27 per cent respectively below their initial values.   These findings correspond with those found by McDowall (5) and by Roome (4).   Following the cessation of hyperventilation, periods of apnea lasting from 60 to 100 seconds were observed in all cases.

The degree of alkalosis achieved is indicated by the increases found in the serum pH.   As was to be expected, the serum $(BHCO_3)$ decreased markedly and the serum chloride increased slightly.

Effects of Respiratory Acidosis (Table II)—Since the animals were under barbital anesthesia, the respirations increased gradu-

ally in rate but not in amplitude. No significant change in blood pressure was observed in this series of experiments.

TABLE I

Changes in Serum and Muscle Following Respiratory Alkalosis

Muscle values corrected for neutral fat.

		pH	(CO₂)	(BHCO₃)	pCO₂	Plasma protein	(H₂O)	(Cl)	(Na)	Total base	(F)

Dog V; weight 16 kilos; time of overbreathing 60 min.; initial blood pressure 120 mm. Hg; blood pressure during overbreathing, 110; per cent of initial blood pressure, 92; period of apnea following overbreathing, duration 60 sec.

		mM per $l.$	mM per $l.$	$mm.$ Hg	$gm.$ per $kg.$	$gm.$ per $gm.$	$m.$-$eq.$ per $kg.$	$m.$-$eq.$ per $kg.$	$m.$-$eq.$ per $kg.$	
Serum	Initial	7.37	24.70	23.44	41.9	55.3	0.9264	108.6	144.6	154	
	Final	7.48	16.10	15.46	21.4	56.7	0.9241	110.0	144.1	154	
Muscle	Initial						0.7744	21.71	31.04		178
	Final						0.7786	21.40	30.45		173

Dog VI; weight 14 kilos; time of overbreathing 60 min.; initial blood pressure 100 mm. Hg; blood pressure during overbreathing, 68; per cent of initial blood pressure, 68; period of apnea following overbreathing, duration 100 sec.

Serum	Initial	7.38	25.85	24.56	42.8	56.0	0.9232	109.1	135.6	153	
	Final	7.72	11.63	11.36	9.1	54.1	0.9242	113.5	138.8	153	
Muscle	Initial						0.7695	27.59	38.91		224
	Final						0.7695	27.04	35.76		211

Dog VIII; weight 18 kilos; time of overbreathing 62 min.; initial blood pressure 148 mm. Hg; blood pressure during overbreathing, 108; per cent of initial blood pressure, 73; period of apnea following overbreathing, duration 90 sec.

Serum	Initial	7.41	28.43	27.10	44.2	60.7	0.9167	107.2	141.8	155	
	Final	7.68	13.40	13.06	11.4	56.0	0.9246	113.7	142.0	155	
Muscle	Initial						0.7649	27.06	38.15		222
	Final		.				0.7679	24.08	35.07		188

The extent of the acidosis produced is shown by the decrease in the pH of the blood serum. Accompanying the acidosis was

the usual increase in serum (BHCO₃) and the slight decrease in the serum chloride.

TABLE II

Changes in Serum and Muscle Following Respiratory Acidosis
Muscle values corrected for neutral fat.

		pH	(CO₂)	(BHCO₃)	pCO₂	Plasma protein	(H₂O)	(Cl)	(Na)	Total base	(F)

Dog I; weight 11 kilos; time of rebreathing 60 min.; initial blood pressure 156 mm. Hg; blood pressure during rebreathing, 158; per cent of initial blood pressure, 100

		pH	mM per l.	mM per l.	mm. Hg	gm per kg.	gm. per gm.	m.-eq. per kg.	m.-eq. per kg.	m.-eq. per kg.	
Serum	Initial	7.43	26.91	25.71	39.9	37.3	0.9259	110.1	138.8	154	
	Final	7.20	32.18	29.81	78.8	51.1	0.9265	109.1	142.0	156	
Muscle	Initial						0.7663	19.63	31.45		158
	Final						0.7584	21.20	32.30		173

Dog II; weight 14 kilos; time of rebreathing 50 min.; initial blood pressure 106 mm. Hg; blood pressure during rebreathing, 100; per cent of initial blood pressure, 95

Serum	Initial	7.39	24.40	23.21	39.6	67.9	0.9150	106.5	136.4	152	
	Final	7.15	30.90	28.37	84.0	68.0	0.9155	104.7	139.0	153	
Muscle	Initial						0.7728	20.07	31.20		166
	Final						0.7619	18.50	27.60		155

Dog IX; weight 12 kilos; time of rebreathing 60 min.; initial blood pressure 160 mm. Hg; blood pressure during rebreathing, 150; per cent of initial blood pressure, 94

Serum	Initial	7.42	25.65	24.48	38.8		0.9225	109.0	139.0	156	
	Final	7.08	31.96	28.93	100.6		0.9235	108.0	142.0	157	
Muscle	Initial						0.7713	23.70	32.50		193
	Final						0.7608	21.55	31.10		179

Changes in Water and Salt Distribution—The analytical data have been subjected to the same mathematical treatment as that described in detail in Paper I of this series (1). The amounts of

extracellular phase (F), in gm. per kilo of muscle, were calculated
from the equation

$$(F) = \frac{(Cl)_M \times (H_2O)_s \times 1000}{1.04 \times (Cl)_s}$$

in which the subscripts M and s represent muscle and serum respectively, and are given in the last column of Tables I and II.

On the basis of these values of (F) the changes produced in the intra- and extracellular phases of 1 kilo of the original muscle have been calculated and plotted in Fig. 1.

The changes observed in the extracellular phase ΔF in the present experiments were, as might have been expected, distinctly less than those found as a result of the injection of large quantities of isotonic salt solutions. It is felt that the changes in ΔF were not sufficiently consistent in direction or magnitude to be of significance. It is probable that whether there is an increase or decrease of the extracellular phase of muscle, following the changes in the pH of the blood, depends on numerous physiological factors such as changes in blood flow, excretion, and the previous state of the animal.

However, when the changes produced in the intracellular phase of the muscle (ΔC) were estimated, it was found that there were consistently an increase in alkalosis and a decrease in acidosis. It may be noted that these changes are opposite in direction to those which occur in red cells, when the pH of the blood is changed by varying the CO_2 tension.

In the alkalosis series the increase in the intracellular phase was apparently largely at the expense of the extracellular phase, although there is evidence in two of the three experiments cited that fluid to the amount of 12 and 13 gm. per kilo was added to the muscle.

In the acidosis series there was apparently an actual withdrawal of fluid from the muscle amounting to 35 to 40 gm. per kilo. It would appear that water had been withdrawn, leading to hypertonicity of the extracellular fluid, which in turn resulted in the withdrawal of water from the intracellular phase until osmotic equilibrium was again established. The changes observed in this series were in the same direction as those found in dehydration experiments which are to be reported subsequently.
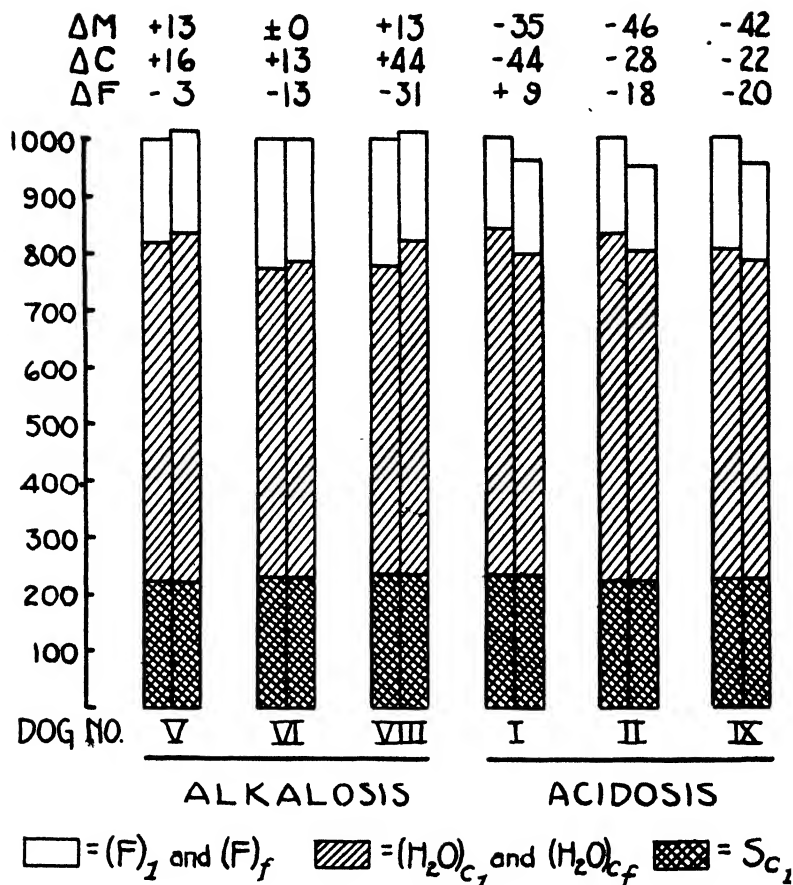
FIG. 1. Intracellular water and solids and extracellular phase before and after experimental procedures, showing volume changes in gm. The first column in each pair presents the original data, and the adjoining column the final data, for one experiment. ΔM represents the absolute change in 1 kilo of muscle; ΔC and ΔF, absolute change in intracellular and in extracellular phase per kilo of original muscle, respectively; $(F)_I$ and $(F)_f$, gm. of extracellular phase per kilo of muscle, originally and finally, respectively; $(H_2O)_{C_i}$ and $(H_2O)_{C_f}$, gm. of intracellular phase per kilo of muscle, originally and finally, respectively; $(S)_{C_i}$, gm. of intracellular solids per kilo of muscle, originally.

SUMMARY

Experiments on normal dogs are described in which the extra-
and intracellular phases of muscle were studied during respiratory
alkalosis induced by overbreathing and during respiratory acidosis
induced by breathing CO_2 mixtures.   From these data it was
concluded that respiratory alkalosis results in an increase and
respiratory acidosis in a decrease in the intracellular phase.

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THE EXCHANGE OF SALT AND WATER BETWEEN MUSCLE AND BLOOD

III. THE EFFECT OF DEHYDRATION*

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The subject of dehydration has assumed increasing prominence of late owing to its importance in certain physiological and clinical problems. The recent work of Darrow and Yannet (1) has done much to clarify the course of events which results from the addition of salt, on the one hand, and the addition of water, on the other. From the analyses of serum and cells, following the intraperitoneal injection of hypertonic NaCl or isotonic glucose, they concluded that, under the conditions of their experiments, tissue cells shrink in the former instance and swell in the latter.

Dehydration is usually thought of as referring to loss of water by the organism or its component parts. In view of the fact that it has become possible to estimate the relative amounts of intra- and extracellular phases of muscle (2), a study has been made of the changes produced in these phases under various experimental conditions which are known to lead to dehydration.

Data will be presented illustrating several different experimental conditions which come under the category of dehydration. These data will illustrate that, depending upon the method of dehydration employed, there may be a decrease in both the intracellular phase and the extracellular phase, there may be a decrease in the intracellular phase and a considerable increase in the extracellular phase, there may be no decrease or even a slight increase in the intracellular phase with a decrease in the extracellular phase.

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* A report of the work discussed in this paper was presented at the meeting of the National Academy of Sciences in Chicago, November 16, 1936.

General Considerations

Before presenting the experimental work, the changes which could be brought about in the intra- and extracellular phases of muscle by reasonable changes in the salt and water of muscle will be considered, in order that the meaning of the experimental data may be clearer. For purposes of this discussion, it will be as-
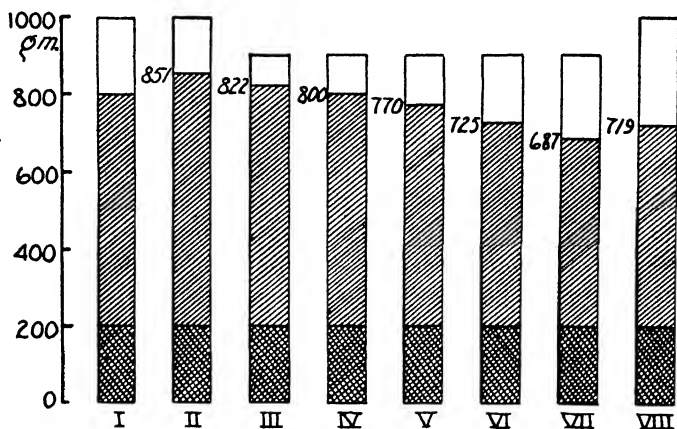
Fig. 1. The relative amounts of extra- and intracellular phases of 1 kilo of muscle following various hypothetical conditions of dehydration. Column I presents the average values for 1 kilo of normal muscle, and each of the succeeding columns presents a change in the salt and the water of the muscle, with corresponding results. The clear areas represent the extracellular phase; the single cross-hatched areas, the water of the intracellular phase; and the double cross-hatched areas, the solids of the intracellular phase.

sumed that the intracellular phase is impermeable to proteins and salts, but permeable to water. The various hypothetical conditions have been diagramed in Fig. 1, and the following discussion will refer to these conditions.

Normal State—Column I (Fig. 1) represents diagrammatically the relative amounts of the extra- and intracellular phases of 1 kilo of average normal muscle and the water distribution in these phases.

The extracellular phase is assumed to be 200 gm., consisting of 99 per cent H_2O, and the intracellular phase 800 gm., consisting of 600 gm. of water and 200 gm. of solid. The concentration of osmotically active substances is assumed to be 320 mM per kilo of H_2O in both phases. (This would correspond to a total base concentration of 160 milli-equivalents per kilo of H_2O.)

The subsequent discussion will be concerned with what changes would occur to restore equilibrium if one removed or added salt and water, singly or together, from the extracellular phase of this kilo of muscle.

Salt Abstraction without Water Change—If one conceives of the osmolar concentration of the extracellular phase being reduced by an amount equivalent to the removal of 10 mM of NaCl, without a change in the water content of the muscle, the resulting relations would be as shown in Column II. The extracellular phase has decreased 51 gm. and the intracellular phase has increased 51 gm., owing to the transfer of water from the former to the latter until the osmolar concentrations in the two phases are again equal—in this case, 295 mM per kilo of H_2O. This is an instance of dehydration in the sense that the extracellular phase has been diminished. It has been illustrated for the body as a whole by Darrow and Yannet's experiments on the consequences of the intraperitoneal injection of isotonic glucose.

Removal of Hypertonic Solution—Column III illustrates the change produced when salt and water are removed in the proportions of a hypertonic solution. In the example cited 20 mM of NaCl and 100 gm. of water are assumed to have been removed. At equilibrium, one finds the extracellular phase to have decreased from 200 to 78 gm., but the intracellular phase to have increased from 800 to 822 gm. This is an exaggerated example of what happens at certain stages following the injection of isotonic glucose, as our experiments will illustrate.

Dehydration of this type is characterized by the greatest decrease in the extracellular phase, and leads to severe clinical symptoms, dryness of mucous membranes, flaccid skin, and decrease in excretion of urine.

It should be particularly noted that to restore a normal condition in such a tissue would require hypertonic NaCl, not an isotonic solution.

Removal of Isotonic Solution—Column IV shows the effect of the withdrawal of 16 mᴍ of NaCl and 100 gm. of H_2O on the extra- and intracellular phases.   In this case there would be no shift of water and the only effect would be the decrease in the extracellular phase by 100 gm.

Removal of Hypotonic Solution—Column V shows the changes which would be produced by any procedure which had the effect of removing 100 gm. of 0.1 ᴍ NaCl from the muscle.   The original muscle, as a whole, would lose 100 gm. of water, of which 30 would come from the intracellular and 70 from the extracellular phase.   This would be an instance of decrease in the bulk of both the intracellular and extracellular phases.   Experimental examples of this will be given later.

Removal of Water Alone—If 100 gm. of water are removed from the muscle, changes similar to those just related will occur, but obviously in a more exaggerated form.   Column VI shows the quantitative changes which would occur.

Removal of Water and Addition of Salt—If the changes in the muscle constituents are equivalent to the addition of salt and the withdrawal of water, the changes produced in the muscle phases would be those shown in Column VII.   The muscle as a whole has lost 100 gm. of water, the extracellular phase has gained 13 gm., and the intracellular phase has lost 113 gm.   Experimental examples of this type of dehydration will be given below.

Addition of Salt—There may be some question as to whether this type of change belongs in the category of dehydration.   It does, however, illustrate an instance in which the intracellular phase might conceivably decrease in bulk owing to loss of water, and, in that sense, be dehydrated.   It is really a special instance of the previous example; *i.e.*, one in which there is no change in the water content of the muscle as a whole.   This condition has been discussed for the body as a whole by Darrow and Yannet, and has been illustrated in their experiments on the injection of hypertonic NaCl.

From the above considerations, it may be concluded that dehydration of tissue may be of several types, briefly characterized as follows: decrease of extracellular phase with swelling of intracellular phase (Columns II and III), without change of intracellular

phase (Column IV), with shrinking of intracellular phase (Columns V and VI); decrease of intracellular phase with increase of extracellular phase (Columns VII and VIII), without change of extracellular phase (not illustrated), with decrease of extracellular phase (Columns V and VI).

<div align="center">EXPERIMENTAL</div>

This study included two groups of experiments: (1) animals receiving intravenous injections of hypertonic sodium chloride (25 per cent) or sucrose (50 per cent), and (2) animals receiving intraperitoneal injections of hypertonic sodium chloride (1.8 per cent), isotonic sucrose (9.6 per cent), and isotonic glucose (5.5 per cent).

Normal healthy dogs were used as experimental subjects and were anesthetized with sodium barbital as in the preceding experiments (2). After the dog was anesthetized, a cannula was introduced into the femoral artery. 30 cc. of blood were taken under oil, and one of the rectus abdominis muscles was removed, for the control analyses. The animal then received either an intravenous injection of hypertonic solution or an intraperitoneal injection of isotonic or hypertonic solution. All solutions were freshly prepared for each experiment and warmed to 38° for injection. The intravenous injections were given by gravity at the rate of 5 cc. per minute. This slow injection rate avoided hemolysis of the red cells. The intraperitoneal injections were given by gravity into the peritoneum at the rate of 50 cc. per minute, after which varying intervals of time (from 1.5 to 4.0 hours) were allowed to elapse. The opposite rectus abdominis muscle was then removed, and a second sample of blood was withdrawn from the cannulated femoral artery under oil, for the final analyses. The urine and peritoneal fluid were then removed and measured.

The following determinations were made on the serum: pH, CO_2, water, chloride, sodium, and total base; on the muscle: water, chloride, sodium, and total neutral fat; on the peritoneal fluid: protein, chloride, sodium, and total base. All analyses of serum and fluid were made in duplicate; all muscle analyses, in quadruplicate. All muscle analyses were corrected for neutral fat. The technique for the removal and treatment of the blood and muscle and the chemical methods were the same as those

described in Paper I of this series (2). Continuous blood pressure tracings were recorded, by the connection of a cannula in the

TABLE I

Changes in Serum and Muscle after Intravenous Injection of Hypertonic Solutions

Muscle values corrected for fat.

		pH	CO₂	Plasma protein	H₂O	Cl	Na	Total base	(F)

Solution, 25 per cent NaCl, 2.75 cc. per kilo
Dog 60; weight 18 kilos; time elapsed, 1 hr.; urine, 136 cc.

			m_M per l.	gm. per kg.	gm. per gm.	m.-eq. per kg.	m.-eq. per kg.	m.-eq. per kg.	
Serum	Initial	7.30	20.35	56.0	0.9284	105.2	134	141	
	Final	7.18	18.89	47.4	0.9349	137.4	162	171	
Muscle	Initial				0.7693	25.10	31.84		212
	Final				0.7540	38.54	47.50		252

Dog 61; weight 16 kilos; time elapsed, 1 hr.; urine, 76 cc.

Serum	Initial	7.37	25.47	49.2	0.9338	112.1	143	151	
	Final	7.14	22.70	47.3	0.9346	139.0	165	172	
Muscle	Initial				0.7684	26.12	42.9		208
	Final				0.7612	38.80	56.4		250

Solution, 50 per cent sucrose, 16.6 cc. per kilo
Dog 65; weight 18 kilos; time elapsed, 1 hr.; urine, 730 cc.

Serum	Initial	7.46	28.22	62.4	0.9169	107.9	142	152	
	Final	7.34	23.20	62.3	0.9033	102.7	133	144	
Muscle	Initial				0.7627	21.92	35.76		178
	Final				0.7437	23.02	37.50		194

Dog 87; weight 9 kilos; time elapsed, 10 min.; urine, 85 cc.

Serum	Initial	7.48	23.87	52.3	0.9297	109.7	139.7	154	
	Final	7.33	16.30	30.5	0.9218	90.8	113.3	124	
Muscle	Initial				0.7608	19.11	29.17		155
	Final				0.7285	19.25	32.34		188

left carotid artery directly to a mercury manometer, in all of the intraperitoneal experiments.

Results

Effect of Intravenous Injection of Hypertonic Solutions—Table I presents the results of the analyses of serum and muscle following the intravenous injection of (a) 2.75 cc. per kilo of body weight of a 25 per cent solution of sodium chloride, and (b) 16.6 cc. per kilo of body weight of a 50 per cent solution of sucrose. In all of these experiments there were decreases in protein, pH, and CO_2 of the serum, increases in the chloride and sodium of the muscle, and a decrease in total muscle water.

Effect of Intraperitoneal Injection of Hypertonic Solution—Table II gives the results of the analyses of serum and muscle following

TABLE II

Analysis of Serum and Muscle after Intraperitoneal Injection of Hypertonic Solution

Solution, 1.8 per cent NaCl. Muscle values corrected for fat. Dog 77; weight 12 kilos; 970 cc. injected; time elapsed, 2¼ hours; urine, 45 cc.

		pH	CO_2	Plasma protein	H_2O	Cl	Na	Total base	(F)
			mM per l.	*gm. per kg.*	*gm. per gm.*	*m.-eq. per kg.*	*m.-eq. per kg.*	*m.-eq. per kg.*	
Serum	Initial	7.43	27.2	54.1	0.9296	111.3	143	157	
	Final	7.29	21.9	50.3	0.9325	137.4·	162	176	
Muscle	Initial				0.7640	19.4	31.77		155
	Final				0.7484	29.4	40.34		191
Peritoneal fluid (per liter)				3.7		163	176		

the intraperitoneal injection of 1.8 per cent sodium chloride. The changes in the serum are in accord with the results of Darrow and Yannet; that is, the water, chloride, and sodium of the serum increased. In the muscle, the sodium and chloride increased, but there was a decrease in the total water.

Effect of Intraperitoneal Injection of Isotonic Solutions—Table III gives the results of the analyses of serum and muscle following the intraperitoneal injection of (a) isotonic sucrose and (b) isotonic glucose. The serum analyses in these experiments parallel the results of Darrow and Yannet; that is, there was loss of water, increase in protein, and decreases in sodium and chloride. Since our study of muscle changes could be carried out only on

<div align="center">

TABLE III

Changes in Serum and Muscle after Intraperitoneal Injection of Isotonic Solutions

</div>

Muscle values corrected for fat.

		pH	CO$_2$	Plasma protein	H$_2$O	Cl	Na	Total base	(F)

<div align="center">Solution, 9.6 per cent sucrose</div>

Dog 82; weight 14 kilos; 1000 cc. injected; time elapsed, 2 hrs.; urine, 90 cc.

		pH	CO$_2$	mM per l.	gm. per kg.	gm. per gm.	m.-eq. per kg.	m.-eq. per kg.	m.-eq. per kg.	(F)
Serum	Initial	7.42	*		62.3	0.9203	106.1	141	155	
	Final	7.33			76.9	0.8985	89.7	117	132	
Muscle	Initial					0.7695	20.11	32.0		167
	Final					0.7685	15.85	25.9		152
Peritoneal fluid (per liter)					3.8		74.6	91.2	99	

Dog 84; weight 10 kilos; 1000 cc. injected; time elapsed, 2 hrs.; urine, 10 cc.

		pH	CO$_2$							(F)
Serum	Initial	7.43	*		62.9	0.9201	109.4	141	155	
	Final	7.32			65.6	0.9010	77.7	111	128	
Muscle	Initial					0.7678	18.91	28.7		153
	Final					0.7535	13.92	22.9		154
Peritoneal fluid (per liter)					2.7		54.7	70.6	79	

<div align="center">Solution, 5.5 per cent glucose</div>

Dog 74; weight 10 kilos; 600 cc. injected; time elapsed, 1½ hrs.; no urine

		pH	CO$_2$							(F)
Serum	Initial	7.33	28.37	66.5	0.9129	111.0	150	166		
	Final	7.30	22.90	69.6	0.9068	96.0	132	144		
Muscle	Initial				0.7585	16.00	23.95		138	
	Final				0.7537	13.1	16.40		131	
Peritoneal fluid (per liter)				1.94		61.0	75	82		

Dog 76; weight 10 kilos; 700 cc. injected; time elapsed, 2 hrs.; no urine

		pH	CO$_2$							(F)
Serum	Initial	7.38	*		62.0	0.9241	118.0	157	175	
	Final	7.36			72.6	0.9110	105.1	143	161	
Muscle	Initial					0.7728	23.20	35.32		188
	Final					0.7612	18.71	25.80		170
Peritoneal fluid (per liter)					1.58		75.2	90	101	

TABLE III—*Concluded*

		pH	CO₂	Plasma protein	H₂O	Cl	Na	Total base	(F)

Dog 79; weight 6 kilos; 500 cc. injected; time elapsed, 2½ hrs.; no urine

			mM per l.	$gm.$ per kg.	$gm.$ per $gm.$	$m.-eq.$ per kg.	$m.-eq.$ per kg.	$m.-eq.$ per kg.	
Serum	Initial	7.36	*		43.1	0.9272	107.2	141	155	
	Final	7.34			62.1	0.9155	90.65	121	136	
Muscle	Initial					0.7886	31.44	45.80		260
	Final					0.7730	23.87	36.10		231
Peritoneal fluid (per liter)					0.8		73.8	91.7	97	

Dog 81; weight 13 kilos; 1000 cc. injected; time elapsed, 4 hrs.; no urine

Serum	Initial	7.33	*	*	0.9183	106.3	146	157	
	Final	7.30			0.9123	82.1	115	130	
Muscle	Initial				0.7515	20.52	29.95		170
	Final				0.7451	13.32	22.44		142
Peritoneal fluid (per liter)						66.7	82	91	

* In these cases there was insufficient serum to carry out all analyses.

animals under anesthesia, the period of equilibration *in vivo* was necessarily limited, and could not be extended over so long a period as when only blood changes are considered. The experiments cited are for varying equilibration times up to 4 hours, beyond which it was not practical to work under barbital anesthesia. Under these conditions, decreases were found in muscle chloride, sodium, and water.

In all experiments, the concentration of chloride, sodium, and water decreased in the serum and muscle, and, although increasing in the peritoneal fluid, had not reached equilibrium with the serum.

Blood Pressure Changes—The blood pressure was lowered following the injections. This change has been observed by Gilman (3).

Volume Changes Produced in the Muscle Phase

From the data of Tables I to III, and the equations previously given (2), the volumes of the extra- and the intracellular phases before and after the intravenous and intraperitoneal injections of hypertonic or isotonic solutions have been estimated.

The amounts of extracellular phase (F) in gm. per kilo of muscle were calculated from the equation

$$(F) = \frac{(Cl)_M \times (H_2O)_s \times 1000}{1.04\,(Cl)_s}$$

in which the subscripts M and s represent muscle and serum respectively, and are given in the last column of Tables I to III.

ΔM	·59	·33	·74	-118	·62	-5	·57	-21	·51	·70	·25
ΔF	+25	+34	+2	+11	+25	-16	-8	-10	-27	-45	-32
ΔC	-84	-67	-76	-129	-87	+11	-49	-11	-24	-25	+7
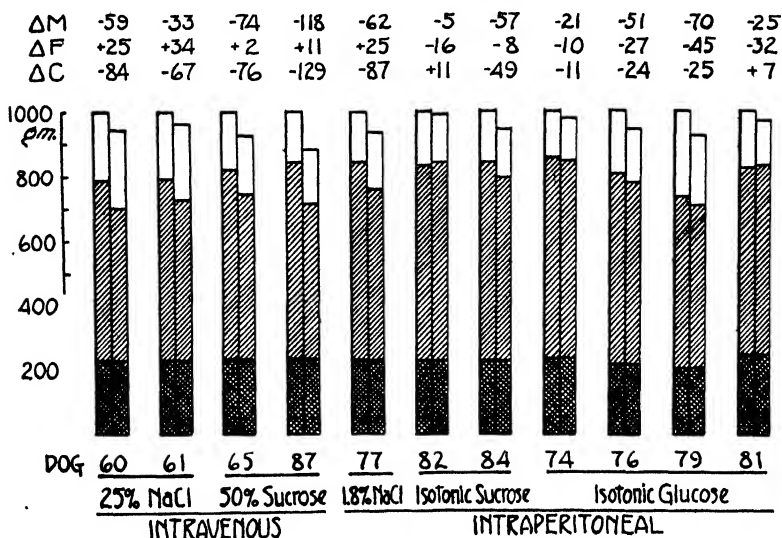
Fig. 2. Intracellular water and solids and extracellular phase before and after experimental procedures, showing absolute change.   The first column in each pair presents the original data, and the adjoining column presents the final data, for one experiment.   The clear areas represent extracellular phase; the single cross-hatched areas, the water of the intracellular phase; and the double cross-hatched areas, the solids of the intracellular phase. ΔM represents the absolute change in 1 kilo of muscle; ΔC and ΔF, absolute change in intracellular and in extracellular phase per kilo of original muscle, respectively.

From the values of (F), the volume changes produced in the intra- and extracellular phases of 1 kilo of the original muscle have been estimated and are graphically presented in Fig. 2.   The changes in the phases produced by the different solutions will be discussed.

Dehydration by Intravenous Injection of Hypertonic NaCl—Hypertonic NaCl injected intravenously led to a loss of fluid by the muscle as a whole (Dogs 60 and 61). This came from the intracellular phase and was removed from the muscle to some other part of the body (the blood stream and the urine among others). An additional amount of water was extracted from the intracellular phase and passed into the extracellular phase, so that the latter actually increased in the experiment.

Such conditions could have been realized by the abstraction of water from the muscle, and by the addition of NaCl to the muscle, corresponding to Column VII of Fig. 1.

The amount of NaCl calculated as being necessary to add per kilo, to realize the experimental results, agrees well with that actually injected per kilo (10.5 mM calculated, compared with 11.7 mM injected).

Dehydration by Intravenous Hypertonic Sucrose—The results observed in these experiments (Dogs 65 and 87) are qualitatively similar to those observed after hypertonic NaCl. The magnitude of the dehydration was greater (as measured by the loss of water by the extracellular phase, and by the loss of water by the muscle as a whole).

The changes could be accounted for by a loss of water from the muscle, and by the addition of a non-diffusible crystalloid (in this case sucrose) in a concentration of 4.5 mM per kilo of muscle.

Dehydration by Intraperitoneal Hypertonic NaCl—In this experiment (Dog 77) there was a loss of H_2O from the intracellular phase amounting to 87 gm. per kilo. Only 62 gm. were removed from the muscle, however, causing a net gain of 25 gm. by the extracellular phase. The water was removed undoubtedly to dilute the hypertonic salt solution which had been injected. There was, at the same time, an addition of salt to the muscle amounting to a calculated value of 9.3 mM per kilo. This experiment also corresponds to the type illustrated by Column VII, Fig. 1.

Dehydration by Intraperitoneal Isotonic Sucrose—The results in these two experiments are not identical. In one experiment (Dog 84) there was loss of water from the intracellular phase amounting to 49 gm. per kilo. From the muscle as a whole 57 gm. per kilo were removed, leading to a net loss of 8 gm. from

the extracellular phase. The calculated change in the non-diffusible crystalloids was negligible. Such a result corresponds to the type shown in Column VI of Fig. 1.

In the other experiment (Dog 82) there was a slight loss of fluid from the muscle as a whole (5 gm. per kilo). There was, however, a loss of diffusible crystalloid amounting to 3.1 mM per kilo. This could be interpreted as meaning that salt was removed from the extracellular phase, leaving the extracellular phase hypotonic. As a result, the intracellular phase increased to restore osmotic equilibrium.

The differences in the results obtained in these two experiments emphasize the fact that during the period that adjustments between the injected solution and the body fluids are taking place, one may expect to find different water changes. This will be brought out even more strikingly in the next section.

Dehydration by Intraperitoneal Isotonic Glucose—The experiments on the injection of isotonic glucose are four in number. They differ from one another primarily in that different lengths of time were allowed to elapse following the injection of the solutions. The elapsed times in experiments on Dogs 74, 76, 79, and 81 were 1.5, 2.0, 2.5, and 4.0 hours respectively. It will be noted that the amount of fluid removed from the muscle progressively increased to 70 gm. after 2.5 hours. This is probably related to the observations made by Schechter *et al.* (4) that the volume of peritoneal fluid increases rapidly within the first 2 to 4 hours after the injection of isotonic glucose. After 4 hours, the fluid loss was less, amounting to only 25 gm. per kilo in the experiment on Dog 81. Up to 2.5 hours, the fluid loss was about equally divided between loss from the extracellular phase and intracellular phase, and could be accounted for by the loss of hypotonic salt solution from the tissue. In the 4 hour experiment, however, there was slight swelling of the intracellular phase. This could be accounted for by the loss from the tissue of 25 gm. of a hypertonic solution, corresponding to 0.228 M NaCl.

DISCUSSION

One has no difficulty in picturing the condition presented after the intravenous injection of hypertonic NaCl. There has been an addition of NaCl to and an abstraction of water from the

muscle.   The water has left the muscle to enter the blood stream; there has been excretion through the kidneys (and probably the gastrointestinal tract); there may even be a diversion of water to the skin and other tissues which have less intracellular fluid phase to draw on than muscle.   Meanwhile, NaCl has been diffusing into the extracellular phase of muscle from the blood stream and leads to the result observed.

When one considers the changes produced by the intravenous injection of hypertonic sucrose, the result is not particularly different.   Owing to the fact that sucrose was not determined in the blood plasma or muscle, we cannot make the same kind of a quantitative comparison between the predicted and observed events.   However, they are qualitatively, at least, in the same direction and of the same order of magnitude.   The diuresis produced in these experiments was greater, and hence the water loss greater.

The intraperitoneal injection of hypertonic NaCl is also in the same direction and consistent with the above discussion.

The situation presented following the intraperitoneal injection of isotonic glucose must be considered in the light of the changes of the salt and water occurring with time.   This has been quite well discussed by Darrow and Yannet (1), Gilman (3), and Robinson and Hegnauer (5), and will be considered here only to the extent that our direct analyses of muscle amplify their results.

The events may be imagined to transpire in some such sequence as the following: Immediately, and for about 2 hours after the injection of the glucose there is migration of ions, principally Na and Cl, from the body fluids to the injected fluid.   This apparently takes place more rapidly than the glucose passes into the fluids and makes the injected solution hypertonic.   Water, therefore, leaves the muscle for the injected fluid to equalize the osmotic pressure.   In our experiments this situation seems to have persisted for at least 2.5 hours.

After 4 hours had elapsed, however, there was a return of water from the injected fluid to the tissue, owing no doubt to the absorption of some of the fluid.   Meanwhile, the combustion of the glucose was proceeding, leaving the extracellular phase of the muscle slightly hypotonic, and leading to swelling of the intracellular phase.

SUMMARY

Experiments on normal dogs are described, in which the volume of extra- and intracellular phases of muscle were studied following (1) the intravenous injection of hypertonic sodium chloride or sucrose and (2) the intraperitoneal injection of hypertonic sodium chloride, or isotonic sucrose, or isotonic glucose. From these data, the conclusions reached were:

1. Following the injection of hypertonic solutions, either intravenously or intraperitoneally, the 1 kilo of the original muscle decreased in volume, with an increase in the extracellular phase and a marked shrinking of the muscle cells.

2. Within 2.5 hours following the intraperitoneal injection of isotonic sucrose or glucose the original kilo of muscle decreased in volume. Both the extra- and intracellular phases lost water during this period.

The significance of various types of dehydration in tissues is discussed.

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STUDIES ON OXIDATION-REDUCTION

XXIII. ASCORBIC ACID [*]

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One of the outstanding chemical properties of ascorbic acid is its reducing ability.   Whether this property is of biological significance is by no means clear, though some evidence has accumulated that similar reducing properties may be important in the control of metabolic processes.   One step towards clarification of this issue would be the determination of the oxidation-reduction potential of the system of which ascorbic acid is the reductant. Previous workers have reported extremely discordant results. Some workers (16, 20, 23), failing to obtain interpretable potentials from mixtures of the oxidant and reductant, have reported the potentials given by the reductant alone.   Such values are thermodynamically meaningless.   Others (13, 14), reporting values for the system in the neutral pH range, give no consideration in interpreting their results to the lability under such conditions of the first oxidation product, dehydroascorbic acid.   Borsook and Keighley (5) were the first to report values free from the above objections.   These workers apparently encountered no difficulties in obtaining an equilibrium between the electrode and the mixture, such as was reported concurrently by Green (16). The work to be reported here had begun at this time and it was the experience of the author then that, though potentials could be established for mixtures, their poor reproducibility left considerable doubt as to their authenticity.   Wurmser and de Loureiro (30) subsequently reported similar experiences concerning the sluggishness of the system.   They were able to obtain, however,

[*] A preliminary report was made before the American Society of Biological Chemists at Washington, March 25–28, 1936.

four values in the acid range in good agreement with those reported by Borsook and Keighley.   In view of these facts it is not surprising that there exists a diversity of opinion with regard to the thermodynamic reversibility of this system.

It will be shown in this paper that the difficulties that have been encountered in measuring this system are entirely due to the facts that the system is electromotively sluggish and that the first oxidation product, dehydroascorbic acid, becomes increasingly unstable as the pH increases above 5.0.   By employing an electromotively active dye system to act as a mediator between the electrodes and the ascorbic acid system it has been possible to record for the first time complete titration curves in the acid region that leave no doubt as to the true thermodynamic reversibility of the ascorbic acid system.   In fact assay of vitamin C in orange juice by the determination of the characteristic titration curve of ascorbic acid has been accomplished.   Since the use of a mediator permits the rapid establishment of the potential of the system at the electrode, it has also been possible to obtain values in the hitherto unmeasured, biological, pH range with concomitant data on the kinetics of the disappearance of the oxidant, dehydroascorbic acid.

Procedure and Results

A commercial sample of ascorbic acid obtained from natural sources was used throughout this work.   Its titration values against both alkali and iodine agreed exactly with the theoretical.

Dissociation Constant—Since the system under investigation was erratic in its behavior, it seemed worth while to establish with certainty any of its characteristics that could be determined by independent means.   One such characteristic that can be used as a landmark is the acid dissociation constant of the reductant. This constant has been determined by measuring the pH of solutions of ascorbic acid which were carefully half neutralized with NaOH and in which the ionic strength was varied by the addition of KCl.   Determinations were made with the hydrogen electrode; the Clark type shaking vessel and a saturated KCl-calomel half cell standardized against both a 0.1 M KCl-calomel half cell and standard acetate buffer, as described by Clark (7) were used.   Clark's tentative standard of potential was used.   The

data and calculations of pK′ for 30° are given in Table I. The values for pK′ were calculated by means of Equation 1:

$$pK' = pH - \log \frac{[B^+] + (H^+)}{[S] - [B^+] - (H^+)} \tag{1}$$

where $[S]$ is the total ascorbic acid concentration, (H^+) the measured hydrion activity, and $[B^+]$ the concentration of base matched against ascorbate ion, here $[B^+] = 0.5\ [S]$. Each pH value is the average of at least three closely agreeing determinations.

TABLE I

pK′ of Ascorbic Acid at Different Ionic Strengths (μ)

Temperature 30.0°.

Total μ	Total ascorbic acid*	Total KCl	pH	pK′†
.	M	M		
0.0011	0.0020	· ..	4.252	4.204
0.0051	0.0101		4.187	4.176
0.0551	0.0101	0.0500	4.113	4.100
0.1051	0.0101	0.1000	4.086	4.072
0.2052	0.0101	0.2000	4.050	4.035

* Half neutralized with NaOH.

† pK = 4.21 (at $\mu = 0$ by extrapolation).

When pK′ is plotted against $\sqrt{\mu}$ the data approximate the curve represented by Equation 2:

$$pK' = pK - \frac{0.5\ \sqrt{\mu}}{1 + 0.5\ \sqrt{\mu}} \tag{2}$$

where pK is assigned a value of 4.21 as a result of extrapolation. This limiting equation is derived from the original Debye-Hückel equation by arbitrarily assigning the term α, the mean ionic diameter, a value of 1.5 Å. Assuming small changes in temperature to have a negligible effect on dissociation constants, the values reported here are in good agreement with that reported by Kumler and Daniels (22). These authors report pK′ = 4.12 at 22–23° and at an ionic strength that can be calculated to be 0.0269. On the same assumption, however, the present data are 0.14 units lower than that reported by Birch and Harris (4) who

find $pK' = 4.17$ at 16–18° where μ apparently has a value of 0.231. Karrer, Schwarzenbach, and Schöpp (20) report a value about 0.12 units higher than that given here. These workers find $pK' = 4.07$ at 24° and an ionic strength of 0.0016 from which they calculate $pK = 4.10$.

In the determination of the oxidation-reduction potentials to be reported later an ionic strength of about 0.10 existed. The value for pK' under such conditions is 4.07. This means that an inflection in the E'_0-pH curve of the ascorbic acid system should be encountered near pH 4.07. Actually such an inflection was found at pH 4.04.

It is of interest to estimate the pK' value of ascorbic acid under biological conditions. Van Slyke, Hastings, Murray, and Sendroy (29) estimate the ionic concentration of blood to be 0.16. The pK' value of ascorbic acid at 30° and $\mu = 0.16$ is 4.05 when calculated by means of Equation 2. If ascorbic acid behaves like other weak acids, approximately the same value should hold for 38°.

Oxidation-Reduction Potentials—The usual titration procedure was found unsatisfactory. Potentials drifted toward "plateau" values that obeyed theoretical relations sufficiently well to be tantalizing, but the rates of drift were slow and depended so much on conditions as to leave a good deal of uncertainty regarding the significance of values judged to be final equilibrium potentials.

In the preliminary experiments it happened that titrations with ferricyanide in phosphate buffer, pH 3.0, gave much more rapid electrode adjustment than when other oxidizing agents or buffers were employed. Under these conditions it was possible to obtain a complete titration curve of ascorbic acid in good agreement with the theoretical. By use of this empirical observation it was possible to show that slowness of electrode adjustment could not be attributed to slow interaction between ascorbic acid and any one of several oxidizing agents, to the nature of the electrode metal, to poisoning of the electrodes, or to the absence of ascorbic acid oxidase (Szent-Györgyi (28)). Associated with the peculiar conditions under which ferricyanide gave good titration curves was an observable amount of decomposition of this iron complex. These results suggested the participation of a mediator. When mediators of known characteristics were introduced, satisfactory

potentials were reached fairly rapidly. It is thus evident that all that is necessary to measure the system is the presence of a small amount of electromotively active substance which when in equilibrium with the ascorbic acid system can impart to the electrodes the potential characteristic of this system.

The most suitable mediators for such a purpose are oxidation-reduction indicators such as those described in earlier papers of this series. As pointed out by Lehmann (24), if the potentials recorded are to be predominantly determined by the system being measured, the mediator must be present in relatively small amounts. It is therefore necessary that the potential of the mediator system lie close to that of the system under measurement, if the maximum poising effect of the mediator is to be realized. An example of the results obtained following these principles is given in Table II. Here the mediator, thionine, is present in a concentration 1 per cent, or less, of that of the ascorbic acid. The normal potential of the thionine system at this pH is $+0.165$ (Clark, Cohen, and Gibbs (9)). No corrections have been applied to the results for acid formed during the oxidation or for the oxidizing power of the mediator, since calculations show that these two factors approximately cancel each other. The uniformity of the calculated values of E'_0 attests to the reversibility of the system and to the fact that 2 electrons are involved in the reaction. The value of $n = 2$ was found to hold within the pH range studied.

Summarized in Table III are the E'_0 values of the ascorbic acid system at various pH values. Those values designated with an asterisk were obtained from a titration curve such as is shown in Table II or from the results of a discontinuous titration. All other values were obtained by 50 per cent oxidation of ascorbic acid with the designated oxidizing agent and mediator. In some cases in which ferricyanide was the oxidant, the addition of a mediator was unnecessary, as was mentioned above. The usefulness of copper sulfate as a mediator at pH 3.0 is of interest in connection with the marked catalytic effect of copper on autooxidation of ascorbic acid as reported by several workers, *e.g.* Barron, DeMeio, and Klemperer (3). Iron was the only other heavy metal of several tried that could function as a mediator. The series of values given at pH 4.58 shows that the results ob-

tained are independent both of the type of oxidizing agent and the mediator used.   There is a difference of 35 millivolts between the methylene blue and thionine systems at this pH (9).   Though the results are not shown here, three different samples of ascorbic acid obtained from natural sources, one of which was kindly supplied by Dr. Szent-Györgyi, and also a synthetic sample gave closely agreeing values.   All values, however, given in Table III were obtained on a commercial sample of natural origin.   Since the oxidant, dehydroascorbic acid, becomes increasingly unstable at pH values greater than 5.0, it was necessary to extrapolate

TABLE II

Titration of Ascorbic Acid

Ascorbic acid (0.002 M) in acetate buffer (0.1 M), pH 4.581; 50 ml. plus 1 ml. of thionine (approximately 0.001 M), titrated with $K_3Fe(CN)_6$ (0.04 M). Temperature 30.0°.

Oxidation	$0.03006 \log [S_r]/[S_o]$	E_h observed	E'_0
per cent		*volts*	*volts*
25.37	$+0.0141$	$+0.1224$	$+0.1365$
35.43	$+0.0078$	$+0.1284$	$+0.1362$
45.50	$+0.0024$	$+0.1338$	$+0.1362$
50.55	-0.0003	$+0.1364$	$+0.1361$
60.46	-0.0055	$+0.1417$	$+0.1362$
70.79	-0.0116	$+0.1478$	$+0.1362$
80.75	-0.0187	$+0.1552$	$+0.1365$
90.79	-0.0299	$+0.1670$	$+0.1371$
95.80	-0.0408	$+0.1776$	$+0.1368$
Average.			$+0.1364$

time-potential curves to obtain the values given in this pH range. In all cases the observed relation at constant pH was $-dE_h/dt = C$, which is indicative of a first order reaction as discussed in Paper XX of this series (2).

A measure of the instability of this oxidant is given in the last column of Table III under the heading, $-\Delta E_h/\Delta t$, the change of potential in millivolts per minute.   Values reported are for an initial equimolecular mixture of oxidant and reductant.   In such a mixture a decrease of potential of about 9 millivolts corresponds to the disappearance of one-half of the amount of oxidant originally present.   Hence the so called "half life" of the oxidant, in

minutes, can be readily calculated by dividing 9 millivolts by the given value of $-\Delta E_h/\Delta t$. For example at pH 7.24 and 30° the half life of dehydroascorbic acid is 9.0/1.6 or 5 minutes. Calculation of the velocity constant for the decomposition of the oxidant from these data can be made according to the procedure outlined previously (2). Attempts were made to correlate the change that

TABLE III

Relation of E'_0 and of $\Delta E_h/\Delta t$ to pH

Ascorbic acid concentration 0.002 M. Temperature 30.0°.

Oxidising agent	Mediator added	pH	E'_0	$-\dfrac{\Delta E_h}{\Delta t}$
			volts	*mv. per min.*
Quinone	Indigotetrasulfonate	1.054	+0.3256*	
$K_3Fe(CN)_6$	None	1.752	+0.2829	
"	"	2.156	+0.2600*	
. "	"	2.511	+0.2392	
"	"	2.698	+0.2266	
"	"	3.044	+0.2087*	
Quinone	$CuSO_4$	3.046	+0.2106	
$K_3Fe(CN)_6$	None	3.391	+0.1882	
Quinone	Methylene blue	3.968	+0.1579	
$K_3Fe(CN)_6$	None	4.006	+0.1541	
"	Thionine	4.581	+0.1364*	
"	Methylene blue	4.581	+0.1345	
Quinone	" "	4.581	+0.1333	
"	Thionine	4.581	+0.1339	
"	"	5.188	+0.1148	0.02
$K_3Fe(CN)_6$	None	5.853	+0.0945	0.06
."	"	6.317	+0.0780	0.09
"	"	6.717	+0.0671	0.21
"	Thionine	7.238	+0.0512*	1.6
Quinone	"	8.319	+0.0205	7.4
$K_3Fe(CN)_6$	"	8.566	−0.0118	18.0

* Titration curve (see text).

occurs in $\Delta E_h/\Delta t$, when pH is altered, with a possible dissociation constant of the oxidant (see below). No simple relation was discovered. The absence of values for $\Delta E_h/\Delta t$ acid to pH 5.0 merely indicates that the rate of disappearance of dehydroascorbic is too slow to be significant under the conditions of the experiments here reported.

One measurement has been made of the system at 38° and pH 7.25. Under these conditions the value of E'_0 is $+0.0462$ and $-\Delta E_h/\Delta t$ is 4.6 millivolts per minute. This value for E'_0 is 5 millivolts lower than that found at 30° and a similar pH. The rate of disappearance of the oxidant has increased about 3-fold with the 8° temperature rise. A similar behavior was noted for the oxidant of epinephrine (2). The half life of dehydroascorbic acid at 38° is thus about 2 minutes.

In the alkaline region the oxidant, dehydroascorbic acid, becomes so unstable that measurements are impossible by the cell technique used to obtain the results presented in Table III. Attempts were therefore made to measure the potential of this system in this region by means of the flow apparatus developed in this laboratory for unstable systems (2). The results were not conclusive. The electrodes do not respond sharply even though the system is apparently more electromotively active in this pH range. The use of mediators did not improve the results significantly. The best results were obtained when a preformed mixture of oxidant and reductant in a weakly buffered acid solution was run against alkaline buffers. Typical results are given on the ascorbic acid curve of Fig. 2 where a dotted line has been drawn through the experimental points to indicate that the data must be considered tentative. The increase in the slope of this curve at about pH 9.4 would normally indicate the dissociation of a hydrion from the oxidant at this point. It should be noted, however, that the dotted portion of the curve does not conform to the 0.06 slope theoretically predictable on the basis of such a dissociation. Since such a hydrion dissociation suggests hydration of the oxidant in the first place, it is possible that the potentials recorded are the resultant of both a dissociation and a dehydration attendant upon such a dissociation. The effect of hydration of the oxidant upon the potentials will be discussed later.

Confirmation of such a dissociation constant for dehydroascorbic acid was sought by colorimetric measurement of the pH of a half neutralized solution of this oxidant. These experiments were performed as follows: The oxidant was formed by complete oxidation of ascorbic acid with ferricyanide or bromine water in a weak HCl solution in which the oxidant is stable. This solution plus different acid-base indicators was mixed with a NaOH solu-

tion in the flow apparatus. The concentration of the latter solution was so adjusted that, when equal amounts of the two solutions were mixed, the oxidant was half neutralized, due allowance being made for the HCl originally present and the acid formed as a result of the oxidation, *e.g.* HBr. The results indicate a pK' value of 8.8 to 9.0 for dehydroascorbic acid. The disappearance of dehydroascorbic acid is apparently accompanied by the formation of a product more strongly acid than its precursor, since the pH values rapidly become more acid.

The electrode equation, stated with numerical coefficients for 30°, that describes the behavior of the ascorbic acid system over the pH range 0 to 8.5 is

$$E_{\hbar} = E_0 + 0.03006 \log \frac{[S_o]}{[S_r]} + 0.03006 \log [K_1(H^+) + (H^+)^2] \qquad (3)$$

Because of the uncertainty of the results in the alkaline region, it seems advisable to limit the equation to this range for the present. In Equation 3, $[S_o]$ and $[S_r]$ are the molar concentrations of the total oxidant dehydroascorbic acid and the total reductant ascorbic acid; (H^+), the hydrion activity. The normal potential E_0 is $+0.3895$ and K_1 is 9.1×10^{-5} at 30°.

Assay of Ascorbic Acid—Knowing the characteristics of the ascorbic acid system, we might consider feasible an accurate assay of the total ascorbic acid content of a natural product as distinct from the total reducing capacity. Orange juice appeared to be the simplest material for the test of this premise. The results of such a test are shown in Fig. 1. Here 10 ml. of orange juice were buffered with 40 ml. of acetate buffer, so that thionine or methylene blue could be used as a mediator. The dye solution should be freshly prepared. The pH of the mixture was determined by the hydrogen electrode and the titration was carried out in a manner similar to that performed on pure ascorbic acid in an oxygen-free environment. After each addition of ferricyanide the potentials reached a steady value within a half hour. These values are represented by the circles in Fig. 1; the smooth curve is the theoretical one for ascorbic acid at this pH. The agreement is very satisfactory. Following the treatment given by Sullivañ, Cohen, and Clark (27) for the indigosulfonates, the dotted curve in Fig. 1 represents the distortion of

the symmetrical theoretical curve that would result if another reversible system with a normal potential 0.040 volts lower was present in a concentration of only 5 per cent of the total.   A similar distortion of the upper half of the curve would be caused by a system with a normal potential 0.040 volts higher and of like concentration.   One may conclude that ascorbic acid is the only re-
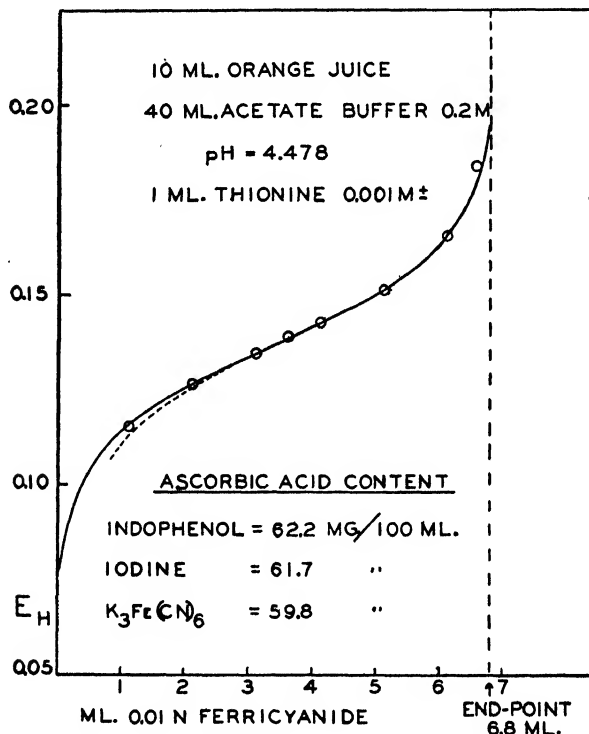
FIG. 1. Content of ascorbic acid in orange juice by electrometric titration with potassium ferricyanide.   Temperature 30°.

ducing material reacting with the ferricyanide or that, if another system is present, its potential lies very close to that of the ascorbic acid system.   The former interpretation seems most reasonable.   On this basis the total ascorbic acid in this sample of orange juice, calculated from the end-point of the titration curve, is 59.8 mg. per 100 ml.   The values determined by titration at

the same pH with 2,6-dichlorophenol indophenol and with iodine are given in Fig. 1. The agreement indicates that these reagents are specific for ascorbic acid when applied to orange juice at this pH. It does not follow, however, that they are also specific when used on other biological materials. It is hoped that similar studies can be made on a variety of such materials to settle this point.

This titration curve also gives the additional information that there is no dehydroascorbic acid in orange juice. The presence of this oxidized form in any appreciable amount would distort the curve; in the graphic description of the titration the initial reading would have been placed on the flat part of the theoretical curve. Thus both the oxidized and total ascorbic acid content are readily determinable by this method.

DISCUSSION

The primary oxidation product of ascorbic acid (I) may be represented as the triketo compound (II). Herbert et al. (19) state that this compound probably exists in aqueous solution mainly in the hydrated form (III). Among other theoretical possible forms of these compounds the two most pertinent to the discussion here are the open chain acids which may be represented by formulas (IV) and (V).

I   II   III

```
        COOH              COOH
         |                 |
   HO—C               O=C                     \
         ‖                 |                    \
   HO—C               O=C                      \
         |                 |
       H·C·OH            H·C·OH
         |                 |
     HO·C·H            HO·C·H
         |                 |
       CH₂OH             CH₂OH

        IV                V
```
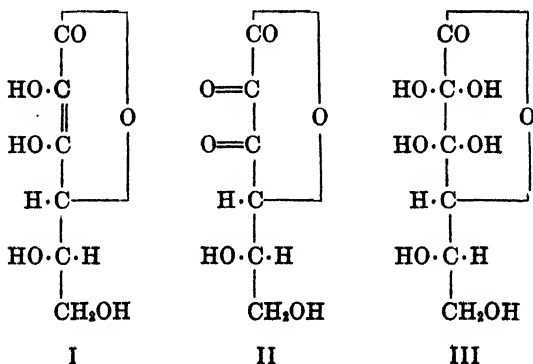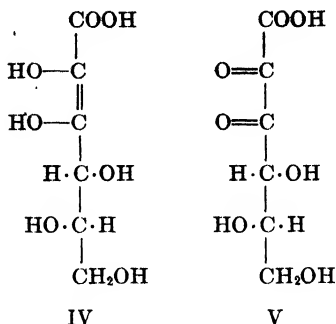
If any or all of these forms are in mobile equilibrium, the free energy change between any pair of oxidants or any pair of reductants would be automatically integrated with the free energy changes observable potentiometrically, so that independent data would be necessary to detect the "tautomers" (see Clark and Cohen (8)). On the other hand, Fieser and Fieser (12) noted that a characteristic of a tautomer, such as a group capable of ionization within the observable pH range, may make itself felt. Now some workers (22, 30) have expressed the opinion that the structural formula (I) proposed by Herbert et al. (19) is not in keeping with the fact that its first acid dissociation constant is of the magnitude characteristic of a carboxyl group. In (IV) and (V) a carboxyl group does appear. Since it is common to both a reductant and an oxidant of the system and since the two ionization constants probably do not differ greatly, no distinct change in the slope $-\Delta E_h/\Delta\text{pH}$ of such a system would be observable. As shown in Fig. 2, however, the ascorbic acid-dehydro-ascorbic acid system changes at about pH 4.0 from a "0.06 slope" to a "0.03 slope" which persists at least to pH 8.6. This indicates that only the reductant contains a group ionizable within this range. Therefore, if this hydrion dissociates from a carboxyl group in the reductant, this group must be destroyed in the reversible oxidation process. This seems unlikely. It is more reasonable to assume that the predominant acid group of the reductant is not a carboxyl as the excellent evidence of Hirst and his coworkers has already indicated. In this connection it may

be pointed out that ascorbic acid contains the grouping $O=C—$
$C=C—OH$ which as Claisen (6) first showed resembles a carboxyl
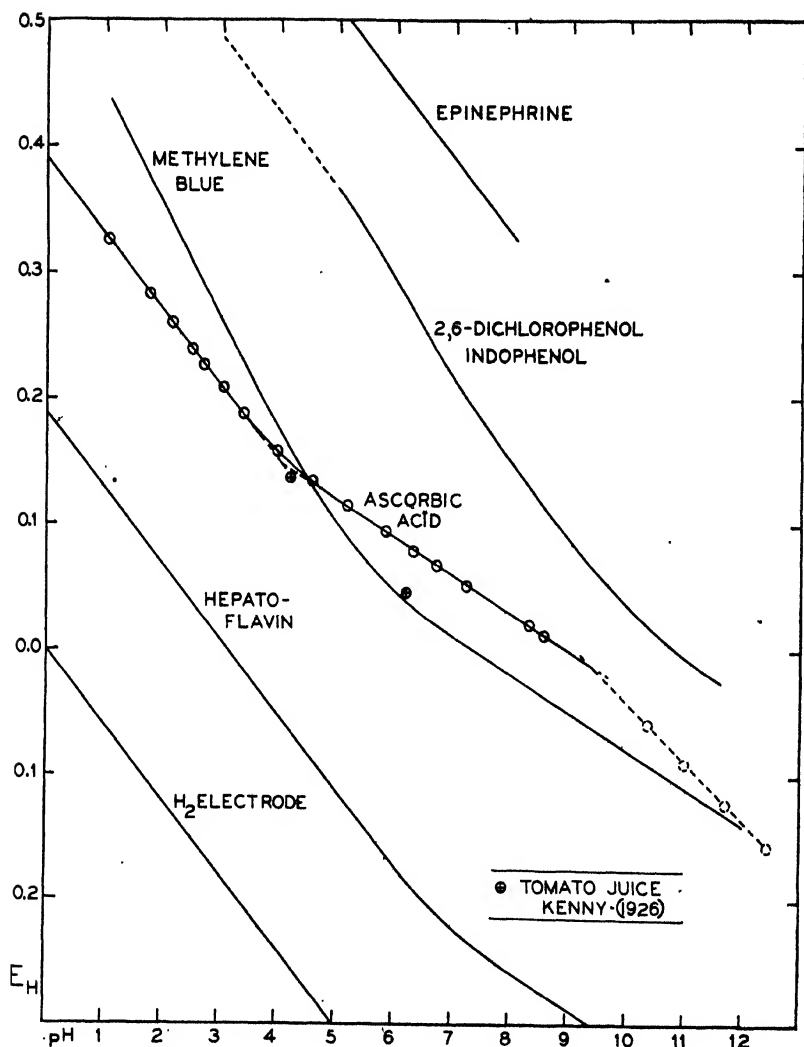
FIG. 2. E'_0-pH curve of the ascorbic acid system in relation to other systems. Temperature 30°.

group.   It has been shown in previous publications (1) that hydroxynaphthoquinones which possess this grouping exhibit acidic properties.   Fieser and Fieser (12) have also shown that 2-hydroxy-1,4-naphthoquinone has a pK' value of 4.05, a value almost identical with that of ascorbic acid under similar conditions. The ring structure present in both these types of compounds undoubtedly enhances the acidic properties of the fundamental grouping.

In this work the term dehydroascorbic acid has therefore been used to designate as oxidant of the system a compound such as (II) or its hydrated form (III).   The data presented here do not permit any definite conclusion to be drawn as to whether hydration of the oxidant occurs or not, for the reasons noted above. It can be concluded, however, that, if a hydration constant is involved, it is not a function of pH within the range 1.0 to 8.6, since to the extent that apparent equilibrium potentials are reliable, the data are accounted for by Equation 3 in which occur only dissociation constants attributable to acid groups already discussed.   It is gratuitous, perhaps, to suggest without independent evidence that hydration, as for instance in (III), may remove a more electromotively active oxidant, such as (II), and thus account for the sluggish response of electrodes without a mediator.

There remains the possibility that a given set of equilibrium data may include those reversible changes that are relatively labile and relatively dominant under the given conditions and test, while a change of conditions may alter the sets of isomers brought into play.   Now the chief condition altered has been pH and the effects have been of three categories: inflection of the E'_0-pH curve, rapidity of electrode adjustment, and change of apparent equilibrium potential attributable to progressive disappearance of an oxidant.   Increase of pH has an effect upon the stability of the system that is so profound as to suggest the throwing into prominence of some unstable tautomer.   The disappearance of dehydroascorbic acid that occurs at increasingly greater rates as the pH becomes greater than 5.0 may be associated with the opening of the lactone ring.   The production thereby of an open chain form such as (V) was first proposed by Herbert *et al.* (19). The acid character of the product so formed has been noted.

Now it has been stated above that if both the oxidant and reductant of the system possessed an acid group of about equal strength, a 0.06 slope would persist into the neutral pH range. As a result the system would exhibit lower potentials in this region. Hence, if such a modification of the oxidant is thrown into play by opening of the lactone ring, its effect upon the potentials is obvious. This would be in agreement with the theory of Fieser that "the tautomer of lower oxidation-reduction potential is the one which will predominate in the equilibrium mixture." Though further data are needed to substantiate such an argument, the experiments of Fruton (13) may be cited for the present as being suggestive.

Certainly it seems likely that very little dehydroascorbic acid exists in the tissues of warm blooded animals, since the half life of this compound is 2 minutes under such conditions. This being the case, it is difficult to see how ascorbic acid can play the rôle of a cyclic catalyst or hydrogen transport in a manner similar to that postulated for such stable systems as the flavins or other pigments. Indeed, if it does, the reaction must be extremely rapid. It should be noted that Szent-Györgyi (28) first proposed such a rôle for ascorbic acid in plant tissue in which a higher acidity and a lower temperature are more favorable to such a mechanism. If, however, the disappearance of dehydroascorbic acid is accompanied by the formation of a species such as (V), which can be converted back into ascorbic acid by the body, we may envision a cyclic catalyst of a different type. Discussions of the peculiar merits of such a catalyst are at present gratuitous.

That an oxidant such as (V) can exist seems fairly conclusive from the work of Herbert et al. (19). It is apparently fairly stable in neutral solutions when protected from further oxidation. In the presence of air or alkali, particularly both, further deep seated changes occur, resulting in a disintegration of the molecule. Of this type perhaps is the well known destruction of vitamin C in natural products by uncontrolled oxidation and heat. Hirst and coworkers have shown that regeneration of ascorbic acid from this open chain acid could be accomplished to the extent of 75 per cent by treatment with HI and subsequent evaporation. Mere treatment with H_2S, however, failed to produce any significant

amount of reducing substances, though such treatment is effective in reducing the lactone form.   This latter observation has been fully confirmed by other workers, *e.g.* Barron, DeMeiò, and Klemperer (3).   Certainly in view of this fact the practice of some workers who attempt to measure so called total ascorbic acid, oxidized and reduced, by first treating their biological material with H_2S before determining its reducing capacity is a worthless one as long as the material in its natural state has a pH greater than 5.0.

These facts raise the question, can the body convert an open chain oxidant such as (V) to ascorbic acid?   The first step in such a process might well be its reduction to 2,3-dienol-1-gulonic acid (IV).   Since this acid is the immediate precursor of ascorbic acid in its laboratory synthesis, feeding experiments to test its antiscorbutic properties might be on record.   These would provide evidence for or against the second step in such a conversion, namely ring closure, being carried out *in vivo*.   Surprising to say, however, search of the literature failed to reveal this compound among the many synthetic ones tested for antiscorbutic properties. Apparently the taboo against association of an open chain acid with vitamin C has worked well.   Nevertheless, there are described in the literature numerous feeding experiments which ascribe varying degrees of antiscorbutic potency to an "oxidized form" of ascorbic acid.   Since, unfortunately, no mention is made of pH control in most of these experiments, it is difficult to tell whether an oxidant such as (III) or an open chain product (V) has been fed.   In the majority of the cases the oxidant was undoubtedly predominantly in the lactone form, since it was formed in an acid environment.   In such experiments the possibility exists that simple reduction to ascorbic acid occurs in the stomach before transformation to the open chain oxidant can be effected by the more alkaline tissues.   In view of these facts, the experiments of Roe and Barnum (25) are perhaps the most enlightening.   There seems little doubt that the compound employed in their experiments was largely an oxidant such as (V).[1]

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[1] The fact that these workers have termed their product reversibly oxidized ascorbic acid which in this paper implies the unaltered oxidant dehydroascorbic acid is no contradiction to this statement.   They have based their application of this term on their valuable observation that

Administration either orally or subcutaneously indicated a potency one-fourth as great as that of ascorbic acid. This furnishes some indication that the body can convert this product into ascorbic acid. Admittedly the evidence has weak links and further work along this line is urgently needed.

In Fig. 2 are given the E'_0-pH curves of ascorbic acid and several other systems whose relationship is of interest. The smooth curve labeled ascorbic acid is the theoretical curve represented by Equation 3; the experimental points are designated by open circles. The values reported by Borsook and Keighley (5) at 35° are 7 to 14 millivolts higher than those given here for 30°. The difference in temperature does not seem responsible for this discrepancy, since a determination at 38° gave even lower values than at 30°. Such a lowering of potential with increase in temperature is in agreement with the results obtained with other systems. The four values given by Wurmser and de Loureiro (30) at 23° are higher still, though here a temperature difference may be partly responsible. It is interesting to note that Kenny (21) in 1926 made a study of the vitamin C content of tomato juice in which he determined its total reducing capacity by electrometric titration and reported the oxidation-reduction potentials recorded at the start of such titrations. These values are given in Fig. 2 as crossed circles and are certainly suggestive of vitamin C itself. Since the reduced form is probably present nearly exclusively in tomato juice, the values would be expected to lie below the curve representing a 50 per cent mixture of oxidant and reductant.

Both methylene blue and 2,6-dichlorophenol indophenol have been used in the titrimetric determination of vitamin C. Reduction of methylene blue by ascorbic acid has, however, been shown to be markedly dependent on the pH. The reaction was found by von Euler et al. (11) to be slowest at pH 5.0, increasing in rate as the pH is altered one way or the other. The reason for the more rapid reaction of methylene blue and ascorbic acid in the acid region is obvious from the relation of the potentials of the two systems. The reduction of methylene blue in the neutral

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regeneration of ascorbic acid from this product can be effected by boiling with SnCl₂ in 30 per cent HCl, conditions both favorable to reduction and ring closure.

and alkaline range by the more positive ascorbic acid system is accounted for by the instability of the initial ascorbic acid oxidant at such pH values.   Such phenomena have been described before (2).   It is obvious that determinations with this dye should be carried out at a pH value less than 1.0 to be quantitative.   The reaction between indophenol and ascorbic acid can be accounted for solely on the basis of the difference in oxidation-reduction potentials of their systems.   That no side reactions occur, at least at pH 4.6, is shown by the fact that, when the dye is titrated with ascorbic acid, the potentials recorded conform strictly with those predictable from the work of Gibbs, Cohen, and Cannan (15).   However, since these workers have shown that such dyestuffs become unstable in the more acid regions, the possibilities of side reactions under such conditions that may invalidate its use must not be overlooked.   Also portrayed for comparison is the only other vitamin that is known to belong to a reversible oxidation-reduction system.   The data for the hepatoflavin system are taken from the work of Stare (26).

In a previous publication (2), in which the potential of the epinephrine system was determined, it was shown that the oxidant of this system was extremely unstable, possessing a half life of 0.06 seconds at pH 7.6 and 30°.   It was therefore postulated that epinephrine must be maintained in a reducing environment in the body to be protected from a virtually destructive oxidation. That ascorbic acid could maintain a reducing environment suitable for such a protection of epinephrine can be seen from the relationship of the two systems portrayed in Fig. 2.   The high content of ascorbic acid in the adrenals is suggestive in this connection and has been so commented upon by other workers (17). Also significant, however, is the observation of Deutsch and Schlapp (10) that, though there is a marked diminution in the ascorbic acid content (*i.e.*, reducing capacity against indophenol) of the adrenals of scorbutic guinea pigs, there is but a slight fall in the epinephrine content.   As long as some ascorbic acid remains, little oxidative destruction of epinephrine would be expected to occur.   Here may be cited the experiments of Heard and Welch (18) who found that no oxidation of epinephrine occurred in perfusates of the adrenal gland until the ascorbic acid also present had disappeared.

SUMMARY

Ascorbic acid is the reductant of a thermodynamically reversible system, sluggish in electromotive activity. By the use of mediators the potential of the system has been determined throughout the pH range 1.0 to 8.6. The normal potential of the system at 30° is +0.390 volts. The first dissociation constant of ascorbic acid has been determined at various ionic strengths. The true dissociation constant has been assigned a value of 6.2×10^{-5} (pK 4.21). Values obtained for the potential in the alkaline range were unreliable partly because of the instability of the initial oxidant. Disappearance of the initial oxidant begins at about pH 5.0 and increases rapidly with increase in pH. At pH 7.25 and 38° the half life of this oxidant is 2 minutes. The significance of these facts in relation to the chemistry and determination of vitamin C and also its rôle in biological processes is discussed.

Determination of the oxidation-reduction titration curve of the ascorbic acid present in orange juice has permitted an accurate assay of its vitamin C content and shown that dehydroascorbic acid, its primary oxidation product, is not a natural constituent.

Addendum—Since the submission of this article there has appeared a paper by Borsook, Davenport, Jeffreys, and Warner.[3] These authors claim to have estimated the oxidation-reduction potentials of three separate steps in the oxidation of ascorbic acid. Their first oxidation stage corresponds to the reaction for which potentials are given here. Their data for this step need no comment, since they are apparently the same as those reported earlier by Borsook and Keighley (5) and have been discussed above. (The expression of the results, now as before, in millivolts instead of volts is obviously an error.) Their second oxidation stage involves the oxidation of the product, presumably an open chain acid, resulting from a transformation of the oxidant of the first stage. No evidence was found by the present author to indicate that this product behaved as a reductant in a manner such as to interfere (at pH values greater than 6.0) with the potential measurements of the first stage, as is implied by these workers. The potentials assigned to this second stage are based on colorimetric measurements in which, if we understand correctly, small quantities of dye are swamped with the reductant under test. The results obtained with this type of experiment may be extremely misleading, especially since Borsook and his collaborators offer no proof of the reversibility of the re-

[3] Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., *J. Biol. Chem.*, 117, 237 (1937).

action they profess to measure.   No better example can be given than that presented by ascorbic acid itself, for which a similar procedure leads to conclusions concerning the true potential of its system which are grossly in error.   Indeed this very fact has caused confusion in the literature. Fruton's data are cited by these authors in support of their contention, but, as pointed out above, a different interpretation is also possible.   The same arguments may be directed against their estimation of the potential level of a third oxidation stage.   However, the evidence for this stage is weaker still.   In fact it may be looked upon as evidence against their conclusions regarding the second stage.   It is based upon the observation that there is an increase in the rate at which dyes are reduced by the reductant of the second stage as pH increases.   Rates of reaction do not necessarily depend on differences in energy levels.   It is not argued here that these unidentified components possess no reducing capacity, but rather that deductions based upon the sort of physical measurements that were made and without any identification of components are not convincing.

The present author has raised the question as to whether the transformation product of dehydroascorbic acid is antiscorbutic.   Borsook *et al.* have performed feeding experiments in which they have been the first to recognize clearly the lability of the oxidant of ascorbic acid in relation to pH.   They find that this product is inactive.   It is hoped that feeding experiments may soon be reported from our laboratory on a possible reductant of this product, 2-keto-1-gulonic acid.   The important observations of Borsook and his coworkers on the rapid reduction of dehydroascorbic acid by sulfhydryl compounds are suggestive in connection with any interpretation of the rôle of ascorbic acid as a cylic catalyst.

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DIOXANE AS A REAGENT FOR QUALITATIVE AND QUANTITATIVE DETERMINATION OF SMALL AMOUNTS OF IODIDE

ITS APPLICATION TO THE DETECTION OF IODIDE IN IODIZED SALT

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In continuing their studies on absorption indicator titrations in organic media, the authors (1) chanced upon the fact that dioxane will oxidize small amounts of iodide to free iodine which imparts a yellow to orange color to an excess of the reagent. It was thought that this reaction might possibly form the basis of a new method for the determination of small amounts of iodides.

Although dioxane is widely used as a solvent both in the determination of molecular weights (2) and as a solvent in organic chemistry (3), no reference could be found to its reaction with iodides. Its preparation and constitution have been studied by Paterno and Spallino (4). These authors also studied the reaction of dioxane with Br_2, I_2, HCl, $HClO$, PCl_5, $POCl_3$, and cyanogen iodide. They found that it will react with $KMnO_4$ to yield CO_2 and $H_2C_2O_4$.

No attempt will be made in this paper to review the existing methods for the determination of small amounts of iodides. The colorimetric method finally adopted is far simpler in its application than any titrimetric method could possibly be. A review of colorimetric methods for the determination of iodides is to be found in the book on colorimetric determinations by Snell and Snell (5).

Iodine has been determined colorimetrically by means of the

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violet color of its solution in CS_2 by oxidizing with nitrous acid. Chlorine does not interfere nor does bromine if its concentration is less than 5 times that of the iodine (6). Iodides may be determined in the presence of bromides and chlorides by oxidizing to iodates by boiling with excess bromine water and thus liberating 6 times the original amount of iodine. Starch paste is added to the liberated iodine to give a blue color. This reaction has been used by Turner (7) as the basis for a sensitive method for the detection of small amounts of iodides. This color has been found to be unsatisfactory for colorimetric matching by von Fellenberg (8) and by Remington and his coworkers (9). Iodine gives with o-tolidine a blue-green color similar to that of chlorine. Iodates, nitrites, and chlorides and bromides in amounts greater than 1500 parts per million interfere (10). The action of mercurous chloride may be used to form yellow to greenish black mercurous iodide from a sample of iodide solution and the color produced by its adsorption on excess mercurous chloride then serves for its estimation (11). A method for the determination of iodide in iodized salt (0.0221 per cent as KI) has been developed by Geagley (12) who extracts liberated iodine with CS_2 in phosphoric acid solution.

In the method with dioxane as finally adapted by the authors from 0.1 to 15 mg. of iodide can be determined accurately, but a single reagent is required, and the color developed can be matched directly in a colorimeter without necessitating a separation of two layers of different density. Practically no substances have been found to interfere with the reaction. Its application to the determination of iodides in iodized salt will be presented.

Reagents—Dioxane (diethylene oxide); Berg Chemical Company. M.p. 11.0°; b.p. at 760 mm. 101.3°; d_4^{20} 1.0329. Avoid prolonged contact with or inhalation of this reagent.

0.1 N potassium iodide (reagent grade). 1 ml. is equivalent to 12.7 mg. of I^-.

0.01 N potassium iodide. 1 ml. is equivalent to 1.27 mg. of I^-. Diluted from 0.1 N.

Qualitative Test—1.0 ml. of a solution containing 0.1 mg. or more of I^- is placed in a test-tube. The acidity of the solution is adjusted with HNO_3 or 20 per cent sodium carbonate until the

solution is just alkaline to litmus. 3 ml. of dioxane are added and the solution is warmed on a water bath. 1 to 2 drops of concentrated nitric acid is added to the solution. The immediate appearance of a golden yellow to orange color shows the presence of iodides. Thiocyanates, cyanides, carbonates, sulfides, bromides, chlorides, ferrocyanides, ferricyanides, chromates, and iodates, even when present in fairly large amounts, do not interfere with the reaction.

Quantitative Test—Known amounts of iodide ion, from 0.127 to 12.7 mg. of I^-, were carefully pipetted into small calibrated volumetric flasks (3 ml. and 5 ml.), and made up to volume with

TABLE I

Determination of Iodide Ion

Total volume	Iodide ion present	Iodide ion found	Error
ml.	*mg.*	*mg.*	*per cent*
3.0	0.127	0.125	−1.6
3.0	0.254	0.267	+5.1
3.0	0.508	0.496	−2.5
5.0	1.27	1.24	−2.3
5.0	2.54	2.50	−1.6
5.0	5.08	4.90	−3.5
5.0	6.35	6.37	+0.3
5.0	10.16	9.90	−2.6
5.0	12.70	12.40	−2.3

pure dioxane. The yellowish orange color which developed was matched in a Bausch and Lomb microcolorimeter. A typical set of results is given in Table I.

Determination of Iodides in Iodized Salt—A 2.00 or 3.00 gm. sample of the iodized salt was weighed on an analytical balance and transferred to a centrifuge tube. 5.00 cc. of dioxane and 1 drop of concentrated nitric acid were added. The tube was stoppered, shaken, and centrifuged. The supernatant fluid was carefully poured off and the color matched in the microcolorimeter against a known standard. Results with two samples of salt which contained known amounts of iodides are as follows:

Iodide present per 100 gm. NaCl	Iodide found per 100 gm. NaCl	Error
gm.	*gm.*	*per cent*
0.0254	0.0250	−1.6
0.0318	0.0304	−4.4

DISCUSSION

The reaction of dioxane with iodides to liberate free iodine has been found to take place without interference in the presence of large amounts of ions which generally do interfere with such reactions. For example, 50 mg. of KCNS, 50 mg. of NaCl, 50 mg. of NaCN, 20 mg. of KBr, 50 mg. of KIO_3, 50 mg. of $K_4Fe(CN)_6$, 25 mg. of Na_2S, or 25 mg. of Na_2CO_3 have no effect on the reaction of dioxane with 0.254 mg. of I^-. Dioxane reacts with chromates and dichromates to give a colorless solution. Small amounts of iodides may be determined in the presence of 10 mg. of $K_2Cr_2O_7$ by adding dioxane and 1 drop of concentrated nitric acid, and heating on a water bath for several minutes. A golden yellow to orange color will show the presence of iodides. Bromides present in concentrations greater than 20 times that of the iodide seem to have a slight inhibitory effect on the full development of the color. Ferricyanides interfere somewhat with the quantitative reaction, as they give a lemon-yellow solution after being heated with dioxane and nitric acid. They have little or no effect on the qualitative reaction. The qualitative reaction is as sensitive as the nitrous acid test for iodides and requires fewer reagents with consequently less chance of errors due to impurities contained in the reagents. It is possible to detect 0.02 mg. of I^- in a volume of 3.0 ml. of the reagent. Diethylene glycolmonobutyl ether and diethylene glycolmonoethyl ether have been found to give a similar reaction with iodides, but are not as satisfactory as dioxane.

For the quantitative determination of quantities in the range of from 0.1 to 15.0 mg. of iodide ion, the average error is about 3 per cent. The absolute error in most cases is a negative rather than a positive one. Undoubtedly, better results could be obtained by the use of a photoelectric colorimeter.

The quantitative determination of iodides in iodized salt proves that the reagent may be used successfully in the presence of large

amounts of foreign material. It may also be used for the colorimetric determination of iodides in iodates for which the nitrous acid reaction cannot be used.

The possible application of dioxane for the determination of iodides in biological fluids is being investigated. The reagent has been found to precipitate proteins and its color formation with iodides is not affected by sugar, urea, creatinine, or uric acid.

SUMMARY

A simple colorimetric method is presented for the qualitative and quantitative determination of small amounts of iodides (0.1 to 15.0 mg. of I⁻) with dioxane as the reagent. None of the common ions interferes with the reaction and the method has been applied to the determination of iodides in iodized salt.

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COMPARISON OF METHODS OF EXTRACTION OF THE LACTOGENIC HORMONE*

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Following the discovery of the lactogenic hormone in the anterior pituitary by Stricker and Grüter (1) in 1928, crude extracts capable of initiating lactation in suitable animals were prepared in several laboratories. During 1930 and 1931 Parke, Davis and Company prepared crude sheep pituitary extracts which were used by several investigators including Corner (2), Nelson and Pfiffner (3), and Asdell (4). Turner and Gardner (5) prepared extracts of whole sheep pituitaries which stimulated copious lactation in pseudopregnant rabbits. Because it was a true galactogogue, Turner and Gardner (6) called this hormone *galactin.*

Riddle, Bates, and Dykshorn (7) published two short reports in which they showed that an acid- and alkali-soluble isoelectric precipitate from cattle and sheep pituitaries contained the lactogenic principle, which they called *prolactin.*

Gardner and Turner (8) prepared an active extract from sheep pituitaries by digestion in an aqueous alkaline solution at a pH of 8.4 to 9.6 for 24 to 36 hours at 0°, then discarded all residues except the isoelectric precipitate obtained between pH 7 to 5.5. Riddle, Bates, and Dykshorn (9) prepared active extracts by means of an aqueous acid digest at pH 6.5 to 7.0. They also used 60 per cent alcohol at pH 1.5 to 2.5 and pH 10.5 to 11.0.

Lyons and Catchpole (10) described briefly an extraction method in which 66 per cent acetone (pH about 2.0) is used. A precipitate was brought down by increasing the acetone concen-

tration to between 83 and 90 per cent.   The extract was further purified by its isoelectric precipitation at pH 6.4.   McShan and Turner (11) digested the glands with glacial acetic acid and then precipitated the active material in ethyl ether.

Recently Bates and Riddle (12) recommended that the lactogenic hormone be extracted with 60 to 70 per cent alcohol at pH 9 to 10, followed by precipitation of the active principle at pH 5.5 to 6.0 at an alcohol concentration greater than 86 per cent. The precipitate obtained contained, in addition to the lactogenic hormone, the follicle-stimulating and thyreotropic hormones. Methods were described for further separating and purifying these hormones.

While all methods will produce physiologically active extracts, we were anxious to determine which of these methods were most efficient in removing the lactogenic hormone from the anterior pituitary tissue and obtaining the most potent extracts.   To be able to accomplish this purpose it was first necessary to devise a method of quantitative assay.

Methods of Assay—Riddle, Bates, and Dykshorn (9) recommended the use of 2 month-old pigeons or doves which were standardized for body weight.   This method depended upon the increase in crop gland weight.   They defined a bird unit of the lactogenic hormone as "that amount of prolactin which is equivalent to 1 mgm. of our preparation no. 51 this being also the threshold dose per 150 grams body weight of immature doves or pigeons."   They later specified (12) that only doves of races giving a medium crop gland response were suitable for the determination of the potency without a correction factor.

McShan and Turner (13) pointed out the value of the increase in weight of the pigeon crop gland as a qualitative test, but with the pigeons available the crop weight increase was found to be unsatisfactory for quantitative assay, owing to the variability of birds to the same amount of hormone and the impossibility of obtaining reproducible results in assay.   They suggested that a pigeon unit of the lactogenic hormone be "the total amount of hormone injected during a period of 4 days which will cause a minimum but definite proliferation of the crop glands of 50 $\pm$ 11% of 20 common pigeons weighing 300 $\pm$ 40 gm."   A shallow intramuscular injection is made.   As the route of injec-

tion greatly influences the results, it is important in any assay method to follow the same mode of injection.   If the amount of material available for assay is limited, the subcutaneous or intradermal injection over the crop gland will be found far more sensitive.   In this present study the assay method described by McShan and Turner was used.

EXPERIMENTAL

The anterior lobes of approximately 5 pounds of sheep pituitaries were ground in a meat chopper, dehydrated with acetone, and ground in a mortar until the material would pass through an 80 mesh sieve.   This unextracted desiccated pituitary powder was then assayed very carefully.   It was found that 3.5 mg. contained

TABLE I

Comparison of Methods of Extracting the Lactogenic Hormone

Method	Desiccated pituitary	Total bird units	Lactogenic hormone	Per bird unit	Total units recovered	Hormone recovered
	gm.		*gm.*	*mg.*		*per cent*
Gardner and Turner (1933)	20.0	5,700	2.361	1.77	1,300	23.4
Lyons and Catchpole (1933)	45.5	13,000	4.988	1.08	4,600	35.5
McShan and Turner (1935)	20.0	5,700	1.953	1.02	1,900	33.5
Bates and Riddle (1935)	45.0	12,900	2.470	0.15	16,500	128.1
	1*	6,700	0.763	0.113	6,800	101.3

* Further purification of Bates and Riddle extract.

1 McShan-Turner pigeon unit (1 gm. containing 286 units). The pituitary powder was then divided into a number of batches and these batches were extracted according to the methods previously described.   Before the test samples were extracted, preliminary extraction trials were conducted.

The extract recovered, containing the lactogenic hormone, was dried and again assayed.   The results obtained by each extraction method are presented in Table I.

DISCUSSION

These data indicate that the initial extraction by the aqueous alkaline method is very inefficient in the removal of the lactogenic hormone from pituitary tissue, less than 25 per cent of the hor-

mone being removed. It will be noted also that the potency per unit weight was scarcely doubled. The use of acid acetone or glacial acetic acid, while somewhat better than the aqueous method, neither extracted a satisfactory percentage of the hormone from the pituitary powder nor produced a highly potent preparation. The lack of higher recoveries of the hormone by these methods is believed to be due in part to poor extraction, as indicated by assays of the residues, and in part to losses in the extraction solvents.

On the other hand, the 60 to 70 per cent alcohol (pH 9 to 10) extraction method appears to be very efficient. The residues contained very little active material. Further, there appears to be little loss in the extractive fluids, as recovery of the estimated hormone was over 100 per cent. Whether the apparent recovery of more than 100 per cent of the lactogenic hormone is due to biologic variation inherent in the method of bioassay or whether some other pituitary factor depresses the lactogenic hormone in the whole anterior pituitary powder is a problem which is being given further consideration. A stage of purification of this latter preparation in which the thyreotropic and gonadotropic hormones were removed resulted in a lactogenic preparation of increased potency without an apparent loss of this hormone. The further purification of this extract which is reported to increase the potency from 2 to 5 times has not as yet been carried out.

SUMMARY

With an assayed anterior pituitary powder prepared from sheep, a comparison was made of the efficiency of four methods of extraction of the lactogenic hormone. It was found that the 60 to 70 per cent alkaline alcohol extraction method was superior to either the aqueous alkaline, acid acetone, or acetic acid methods.

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THE USE OF CRYSTALS AS CALCIUM ELECTRODES

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Anderson (1) recently criticized my use of mineral crystals as electrodes. He implied that the potential of the crystal electrode included that of a liquid junction at a leak in the crystal.

However, before such an electrode is used, several controls have been made. After having sealed a crystal on a glass tube, the tube is placed for 2 days with the open end in water. If there should be a leak, the water would rise in the tube. Indeed I have had such leaking electrodes, but I did not use them. After this the supporting tube is filled with a solution of $CaCl_2$ and when not in use the electrode is kept in a saturated solution of CaF_2. If there were a leak in the crystal, the result would be, firstly, a drift of the E.M.F. of the cell; secondly, the E.M.F. would be irreproducible. The electrodes used showed no drift and the reproducibility of the E.M.F. was good.

In our researches on gelatin and milk we used Electrode 35 (*cf.* (2)). The results of the checking of the electrode on different days were as follows:

pCa	E.M.F. of cell				
0	68.5	68.0	68.5	68.0	68.5
1	54.5	53.5	54.5	53.5	
2	41.0	41.0	41.0	41.0	41.0
3	28.5	27.5	28.5	27.5	
4	13.5		13.5		13.5

In the meantime I succeeded in making electrodes which show a difference of 27 to 29 millivolts for each unit increment of pCa, the reproducibility being as good as mentioned above. Particulars will be published elsewhere.

Anderson discusses the influence of other ions on the E.M.F. of the cell. I found this influence and therefore I started with iso-electric gelatin, in which there are no foreign ions, and while the electrode is not influenced by the pH.

In the case of milk we have to do with other ions. I found however that between pCa 0 and pCa 2 a concentration of 0.01 N KCl does not influence the E.M.F.; for example

<div style="text-align:right">E.M.F.</div>

$CaCl_2$, 0.1 N	89.0
" 0.1 " in 0.01 N KCl	89.0
" 0.01 N	61.0
" 0.01 " in 0.01 N KCl	62.0

If the concentration of KCl is increased, its influence is observed. The E.M.F. of the cell increases, and therefore the calculated pCa would be too small. Thus in the case of milk the calculated pCa of Table IV ((2) p. 338) could be too small. This means that the adsorption of calcium ions by milk could still be greater, as mentioned. The conclusion reached is unaltered, though it may be that the adsorption of calcium by milk is stronger.

I also tried the paraffin-pinhole electrode as described by Anderson, but I could not get reproducible results. Neither was it possible to get the same differences of potential between solutions which had been 10 times diluted. I also used a liquid junction in a capillary tube, which was also unsatisfactory.

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ON THE FRACTIONATION OF THE VITAMIN B_2 COMPLEX FROM VARIOUS SOURCE MATERIALS*

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We desire to report briefly certain results from a long series of experiments carried out in the latter part of 1935 and during 1936. It was hoped to obtain a highly potent concentrate of vitamin B_6 free from all other components of the vitamin B group. From the results to be presented, some of the difficulties which may be encountered in this type of work may be readily seen.

Since in order to test for any one factor all other vitamins must be provided in an adequate amount, we made a study of the alcohol-ether liver precipitate factor reported by Elvehjem and co-workers (1), as well as of the "filtrate factor" of Lepkovsky and Jukes (2, 3), to determine the extent of their influence in tests for vitamin B_6. Our results from experiments with the liver precipitate will appear elsewhere (4). With the liver we used, and under our conditions, the cure of florid dermatitis in rats and the growth induced by this material appeared to be due to vitamin B_6 which had been adsorbed during the precipitation process and not to a new factor. Our results and conclusions concerning the filtrate factor are included in the present paper.

Table I gives a summary of the results to be reported, while in Fig. 1 are shown graphically the results of some typical experi-

TABLE I

Fractionation of Vitamin B₂ Complex from Various Source Materials

Diet + 200 micrograms vitamin B₁ concentrate daily	Supplement	Average gain in 4 wks.	Symptoms
		gm.	+
787-B	None	−7.2	2.0
Dec., 1935,	100 mg. *brewers' yeast*	2.2	0.8
and Jan., 1936	Adsorbate from alcohol extract of brewers' yeast ≏ 1.0 gm.	14.8	1.2
	Filtrate from above ≏ 1.0 gm.	1.8	0.2
	Adsorbate + filtrate ≏ 1.0 gm. each	17.2	0.0
	None	−6.2	2.2
	Adsorbate from water extract of yeast residue ≏ 1.0 gm.	−8.6	1.8
	Filtrate from above ≏ 1.0 gm.	−1.4	0.6
	Adsorbate + filtrate ≏ 1.0 gm. each	−3.7	0.6
Jan. and Feb., 1936	*Liver filtrate* (1st preparation) ≏ 3.0 gm.	12.0	0.0
	" " (1st ") ≏ 6.0 "	22.5	0.0
	" adsorbate (1st preparation) ≏ 3.0 gm.	9.1	1.8
	" " (1st ") ≏ 6.0 "	16.3	0.8
	" " + filtrate ≏ 3.0 gm. each	32.9	0.0
	None	0.0	1.0
Feb. and Mar.	*Liver filtrate* (2nd preparation) ≏ 0.5 gm.	22.4	0.0
	" " (2nd ") ≏ 1.0 "	25.8	0.0
	" adsorbate (2nd preparation) ≏ 0.5 gm.	0.0	2.0
	" " (2nd ") ≏ 1.0 "	13.0	1.0
	" " + filtrate ≏ 0.5 gm. each	30.2	0.2
	" " + autoclaved filtrate ≏ 0.5 gm. each	12.6	0.2
	None	−4.0	2.0
Apr.	*Liver adsorbate* (2nd preparation) ≏ 0.5 gm.	−3.0	1.0
	" " (2nd ") ≏ 1.0 "	5.8	1.0
	" " + filtrate ≏ 0.5 gm. each	14.7	0.2
	None	1.5	1.0
May	*Liver filtrate* (2nd preparation) ≏ 0.5 gm.	10.0	0.8
	" " (2nd ") ≏ 1.0 "	12.8	0.8
	None	1.1	2.4
793	*Liver filtrate* (2nd preparation) ≏ 3.0 gm. + 5 micrograms lactoflavin (Gain ceased after 10 days and animals lost weight)	11.4	1.6
	Alcohol-ether liver ppt. ≏ 1.0 gm. added	37.0 (28–56 days)	0.0

TABLE I—*Continued*

Diet + 200 micrograms vitamin B₁ concentrate daily	Supplement	Average gain in 4 wks.	Symptoms
		gm.	+
Aug. and Sept.	*Liver filtrate* (2nd preparation) ⇌ 3.0 gm. + 10 micrograms lactoflavin (Gain ceased after 14 days)	16.4	1.8
	Rice bran filtrate ⇌ 0.5 cc. added	19.8 (28–56 days)	0.2
	None	−3.8	3.6
787-B	*Air-dried ground liver* ⇌ 0.25 gm.	14.4	0.6
June	" " " ⇌ 0.5 "	38.0	0.0
	" " " ⇌ 1.0 "	51.6	0.0
Feb.	*Liver extract* aqueous ⇌ 0.25 gm.	15.0	1.2
.	" " " ⇌ 0.5 "	19.8	0.2
	" " " ⇌ 1.0 "	43.0	0.0
.	None	−3.8	2.0
June	100 mg. *wheat germ oil* (Emerson)	−1.6	1.0
	100 " " " " (Hogan)	−3.0	1.0
	Aqueous extract of *brewers' yeast* ⇌ 300 mg.	16.2	0.6
	" " " " " irradiated ⇌ 300 mg.	17.2	0.6
	None	−1.2	1.8
	"	−0.2	1.6
	20 micrograms *hepatoflavin*	−0.5	1.8
793	None	−13.5	3.0
	20 micrograms hepatoflavin	−8.0	2.2
July	5 micrograms *lactoflavin* + *liver filtrate dialysate* ⇌ 3.0 gm.	21.8	1.6
	5 micrograms *lactoflavin* + dialysate adsorbate ⇌ 3.0 gm.	−4.4	3.6
	5 micrograms *lactoflavin* + dialysate filtrate ⇌ 3.0 gm.	10.2	3.5
	5 micrograms *lactoflavin* + non-dialyzable solution ⇌ 3.0 gm.	1.6	3.7
	5 micrograms *lactoflavin* + non-dialyzable adsorbate ⇌ 3.0 gm.	−8.6	3.7
	5 micrograms *lactoflavin* + non-dialyzable filtrate ⇌ 3.0 gm.	0.4	3.7
	None	−3.8	3.6

Table I—*Concluded*

Diet + 200 micrograms vitamin B₁ concentrate daily	Supplement	Average gain in 4 wks.	Symptoms
		gm.	+
793 and 794 Sept.	None	−10.4	2.7
	1 gm. ground *whole wheat*	11.2	1.0
	Whole wheat extract ≎ 4.0 gm.	8.1	0.6
	5 micrograms lactoflavin + 1 gm. whole wheat	20.2	0.2
	5 " " + whole wheat extract ≎ 4.0 gm.	15.3	0.0
	5 micrograms lactoflavin + whole wheat solution ≎ 4.0 gm.	9.6	0.2
	5 micrograms lactoflavin + whole wheat eluate ≎ 4.0 gm. (Animals lost weight after 28 days)	9.2	0.6
	Whole wheat filtrate ≎ 4.0 gm. added	9.0 (35–56 days)	1.0
	5 micrograms *lactoflavin* + *whole wheat filtrate* ≎ 4.0 gm.	−7.4	3.2
793 Oct. and Nov.	5 micrograms *lactoflavin*	0.6	2.8
	5 " " + filtrate factor concentrate	14.0	2.2
	5 micrograms *lactoflavin* + filtrate factor concentrate + whole wheat filtrate ≎ 6.0 gm.	15.0	2.2
	5 micrograms *lactoflavin* + filtrate factor concentrate + whole wheat eluate ≎ 6.0 gm.	22.2	0.6
	5 micrograms *lactoflavin* + whole wheat eluate ≎ 6.0 gm. + whole wheat filtrate ≎ 6.0 gm.	15.4	0.6
	None	−12.6	3.0
	5 micrograms *lactoflavin*	−8.3	3.0
	5 " " + rice bran filtrate ≎ 0.5 cc. original extract	24.6	0.0
	5 micrograms *lactoflavin* + rice bran eluate ≎ 0.5 cc.	14.8	0.0
	5 micrograms *lactoflavin* + rice bran 2nd filtrate ≎ 0.5 cc.	14.8	1.0

ments. Each experiment reported represents averages from several (usually five or more) rats. The results as shown in Table I are grouped somewhat arbitrarily so as to facilitate comparisons.

The basal diets used as well as the scoring system which we adopted are presented in detail elsewhere (5), but in the interest of clarity, we repeat the description.

Diet 787-B has the following composition: casein 18 per cent, salts 4, filtered butter fat 8, cod liver oil 2, and corn-starch 68. In Diets 793 and 794 the butter fat is reduced to 3 per cent and sucrose included at 73 per cent. The casein for Diets 787-B and 793 was extracted with boiling 95 per cent alcohol under a reflux until the alcohol extract showed no fluorescence when tested with a "black light" (four extractions). It was then extracted twice with cold 60 per cent and once with cold 95 per cent alcohol for 24 hours each. In the case of Diet 794 the casein was washed for 1 week in acidulated water, dried, and then extracted continuously for 1 week in a Soxhlet apparatus with 70 per cent alcohol, the alcohol being changed once during the process.

We scored the dermatitis symptoms as follows: + denotes a mild condition; the fur is greasy, the fur, forelegs, and ears are blood-stained, and the animals have ceased growing. 2+ denotes a more severe condition; the ears are swollen, there is a sore area around the nose and mouth, and the feet are red and swollen; at this state the animals have begun to lose weight. 3+, in this stage the eyes are encrusted, all four feet are raw, and the ears are swollen and hard; the nose and mouth are fairly sore. 4+ denotes the final stage; the entire area around the nose and mouth is raw; the feet and forelegs are edematous and sore, the eyes are entirely closed, the ears have sometimes broken off, and there is bloody urine; the animals have lost 10 to 20 gm. in weight.

All rats were weaned at 21 days of age and were depleted (receiving vitamin B_1 only in addition to the basal diet) until growth ceased and mild dermatitis developed. This required about 4 to 5 weeks for the diet containing corn-starch (Diet 787-B) and 3 to 4 weeks for the low fat, high sugar diets (Nos. 793 and 794). The experimental periods were usually 8 weeks, but since with the sugar diets rats which developed dermatitis usually died after 4 to 5 weeks, we are reporting results for 4 weeks only.

The preparation of all test materials was carried out in a darkened room, and all fractions were stored in dark bottles so as to prevent, as far as possible, destruction by light.
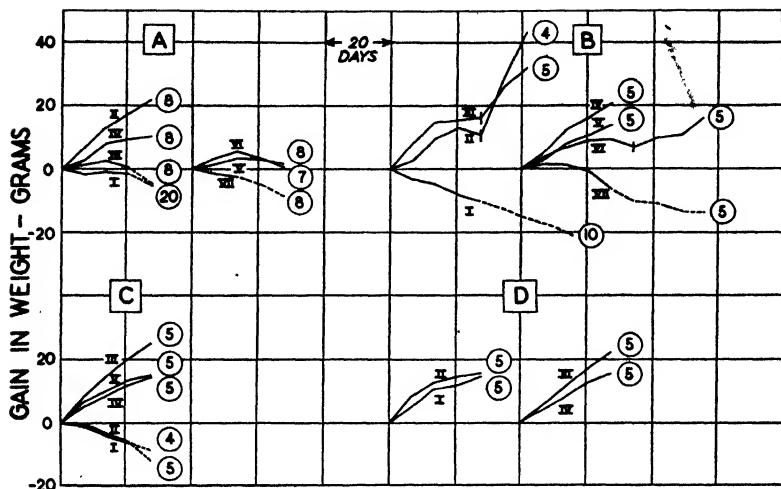
FIG. 1. Curves representing average gain of rats receiving Diets 793 or 794 plus 200 micrograms of vitamin B_1 concentrate daily plus the following.

Section A

		Symptoms +
I. No additional supplement		3.6
II. 5 micrograms lactoflavin + liver filtrate dialysate ⇌ 3.0 gm.		1 6
III. 5	+ dialysate adsorbate ⇌ 3.0 gm.	3.6
IV. 5	+ " filtrate ⇌ 3.0 gm.	3.5
V. 5	+ non-dialyzable solution ⇌ 3.0 gm	3.7
VI. 5	+ " adsorbate ⇌ 3.0 gm.	3.7
VII. 5	+ " filtrate ⇌ 3.0 gm.	3.7

Section B

		Symptoms +
I. No additional supplement		3.6
II. 5 micrograms lactoflavin + liver filtrate ⇌ 3.0 gm.		1.6
Alcohol-ether liver ppt.* ⇌ 1.0 gm. added		0.0
III. 10 micrograms lactoflavin + liver filtrate ⇌ 3.0 gm.		1.8
Rice bran filtrate* added		0.2
IV. 5 micrograms lactoflavin + 1.0 gm. whole wheat		0.2
V. 5 " " + whole wheat extract ⇌ 4.0 gm.		0.0
VI. 5 " " + " " eluate ⇌ 4.0 "		0.6
Whole wheat filtrate* ⇌ 4.0 gm. added		1.0
VII. 5 micrograms lactoflavin + whole wheat filtrate ⇌ 4.0 gm.		3.2

Section C

	Symptoms +
I. No additional supplement	3.0
II. 5 micrograms lactoflavin	3.0

* At the point indicated by the cross line on the curve.

Brewers' Yeast Preparations—Dried brewers' yeast was extracted four times under a reflux with boiling 95 per cent alcohol. The highly fluorescent extract was partially concentrated *in vacuo*, cooled, extracted with ether to remove fat, and then distilled further to remove alcohol and ether. It was acidified to pH 4 and treated twice with fullers' earth, according to the method for adsorption of flavin. The alcohol-insoluble residue was brought to the boil four times with distilled water and the extracts obtained were combined and concentrated, acidified slightly, and treated twice with fullers' earth. For testing, the adsorbates, which had been dried in a vacuum desiccator, were mixed thoroughly with pulverized sugar. The filtrates were fed at about pH 4.

Liver Preparations—For the preliminary series one fresh hog liver was obtained, ground, and immediately extracted four times by just bringing to the boil with distilled water. The combined extracts were concentrated under a vacuum, acidified to pH 4, and treated twice with fullers' earth.

In order to prepare a flavin concentrate, 500 pounds of fresh hog liver were ground and extracted as above. The extract was treated with alcohol so as to precipitate proteins and inert material. It was then filtered and concentrated, acidified to pH 4, and treated twice with fullers' earth. An aliquot portion of the adsorbate was dried and stored in the dark. The filtrate and an

Section C—Concluded

				Symptoms +
III. 5 micrograms lactoflavin + rice bran	filtrate ≈ 0.5 cc. extract	0.0
IV. 5 " " + " "	eluate ≈ 0.5 " "	0.0
V. 5 " " + " " 2nd filtrate ≈ 0.5 cc. extract		1.0

Section D

I. 5 micrograms lactoflavin + filtrate factor concentrate 6 units	2.2
II. 5 " " + " " + whole wheat filtrate ≈ 6.0 gm.	2.2
III. 5 micrograms lactoflavin + filtrate factor + whole wheat eluate ≈ 6.0 gm.	0.6
IV. 5 micrograms lactoflavin + whole wheat filtrate + whole wheat eluate ≈ 6.0 gm. each	0.6

The numeral at the end of each curve indicates the number of animals for which results are included. The dotted line indicates that one or more rats have died. The curve from this date represents gain or loss of surviving animals.

aliquot of the original extract were covered with toluene and stored in the cold (+2.0°), shielded from light. For feeding tests the material was distilled to remove toluene. For autoclaving, the filtrate was distilled, then brought to pH 9, autoclaved for 1 hour, then acidified to pH 4.

Wheat Germ Oil—Two samples of wheat germ oil were used. One was supplied by Professor A. G. Hogan. The other was prepared by Dr. O. H. Emerson in this laboratory by extraction of wheat germ with high boiling petroleum ether, the solvent being distilled off under a vacuum.

Dried Liver—This was prepared from fresh hog liver which was ground immediately and air-dried before a fan.

Brewers' Yeast Extracts for Irradiation—This extract was prepared according to the procedure described by Richardson and Hogan (6). An aliquot was irradiated for 10 hours at a distance of 14 cm. from a mercury arc lamp in an apparatus similar to theirs. The pan was set in a metal jacket through which tap water circulated, and the whole was rocked continuously, thus preventing overheating and allowing thorough mixing.

Hepatoflavin was prepared according to the method described by Lepkovsky, Popper, and Evans (7).

Crystalline lactoflavin used in later experiments was furnished by Vitab Products, Inc.

Dialysis—The dialysis was carried out as follows: The liver filtrate was distilled to remove toluene. About 200 cc. were placed in each of several cellophane tubes (Visking Corporation) in a large covered crock in the cold room. Sufficient distilled water was added to immerse the bags. The dialysate was siphoned out daily and fresh distilled water added. The process was continued until the dialysate was colorless (about 2 weeks). Each day's dialysate was concentrated under a vacuum to a small volume. At the end of the period the dialysates were combined and an aliquot reserved for testing. Since we hoped to adsorb vitamin B₆, we acidified the remaining dialysate to pH 1 to 2 and used larger amounts of fullers' earth (two lots of 50 gm. each for each 1750 gm. equivalent of liver). The non-dialyzable solutions were combined, concentrated, and an aliquot acidified and treated with fullers' earth. The adsorbates were dried in a vacuum desiccator and the filtrates brought to pH 4 for feeding.

Whole Wheat Preparations—The alcoholic extract was prepared

according to the method of Bourquin and Sherman (8). The alcohol was distilled off and the extract divided into three portions. One portion was poured onto a small amount of corn-starch and dried for feeding in a vacuum desiccator (whole wheat extract). The second portion was fed as such (whole wheat solution). The remainder was acidified to pH 1 to 2 and treated with fullers' earth (two lots of 100 gm. each for the extract from 1200 gm. of whole wheat). The filtrate was brought to pH 4 for feeding (whole wheat filtrate). The combined adsorbates were shaken twice with 0.1 M Ba(OH)$_2$ for 20 minutes. The material was filtered as rapidly as possible into a flask containing H$_2$SO$_4$. The resulting BaSO$_4$ was filtered off and the eluate brought to pH 4 for feeding (whole wheat eluate).

Filtrate Factor Concentrate—This was kindly furnished by Dr. T. H. Jukes. It consisted of a liver extract from which all flavin and vitamin B$_6$ had been removed, according to the method described by Lepkovsky, Jukes, and Krause (3). 6 chick units were fed daily.

Rice Bran Filtrate—This was supplied by Vitab Products, Inc. Two adsorptions had been carried out on the original extract to remove flavin and vitamin B$_1$ and it had been concentrated to a thick syrup. Apparently the original extract was exceedingly rich or the flavin and vitamin B$_1$ were in such combinations as to be difficultly adsorbed, since on dilution there was decided fluorescence and, when fed in combination with 100 micrograms of vitamin B$_1$ concentrate to rats receiving the Chase and Sherman vitamin B$_1$-deficient diet (9), the growth rate was doubled. We prepared an adsorbate and eluate and the resulting filtrate according to the method described for the whole wheat concentrates. Since the rats were receiving adequate vitamin B$_1$ and flavin, the presence of these factors in the rice bran filtrate would not influence the results.

DISCUSSION

In general, rats develop dermatitis rather more slowly on Diet 787-B than on the sugar diets. Symptoms to a score of 2 + are as severe as we have obtained in 4 weeks with the former, whereas with Diets 793 and 794 a score of 3 to 4 is obtained in this period of time. Therefore, by comparison of the scores of animals receiving supplements with those of rats receiving vitamin B$_1$ only

or B_1 and flavin, the vitamin B_6 potency can be approximated. According to the work of Lepkovsky and Jukes, rats receiving vitamins B_1, flavin, and vitamin B_6 will not gain unless supplied with the filtrate factor. In cases where animals do not develop dermatitis, yet do not grow, we have assumed a lack of the filtrate factor only.

Applying these criteria to the data presented, we would draw the following conclusions.

Hot alcohol apparently extracts the greater part of the vitamin B_6 and flavin from brewers' yeast, since rats receiving concentrates from this fraction grew better and developed less severe dermatitis than did those which received supplements prepared from the water extract. No filtrate factor appears to be present in this material. Some, at least, of the vitamin B_6 was adsorbed along with flavin.

In the case of the liver filtrate experiments it appears that little vitamin B_6 had been adsorbed, since the potency of the freshly prepared material was similar to that of the original liver extract. There was in every case a better than additive gain when the adsorbates and filtrates were fed in combination, due probably to the filtrate factor. Autoclaving reduces the growth-promoting potency without lowering its vitamin B_6 value. With storage, there is a progressive loss in vitamin B_6 potency. In the experiment carried out in August and September the rats receiving the filtrate with crystalline flavin gained 6 to 8 gm. per week for 2 weeks and then became constant or lost weight rather rapidly, developing dermatitis. Addition of the Elvehjem liver precipitate or rice bran filtrate resulted in rapid cure of the condition, with gain in weight.

Since it was impractical to attempt a test of fresh raw liver, it is not possible to determine the extent of loss in air drying. An amount equivalent to 0.5 gm. of fresh liver as the sole supplement gave complete protection from dermatitis and a gain approximating 1.5 gm. per day. Apparently our method of extraction was efficient, since results with the liver extract are similar to those with the air-dried liver.

Neither sample of wheat germ oil protected the rats from dermatitis. The animals barely maintained their weight, but since the oil was fed as the sole supplement, no gain could have been expected.

In our hands irradiation of the yeast extract did not destroy any of the factors present in the original extract. The growth rates and the condition of the animals receiving irradiated or non-irradiated extracts as the sole supplement were practically identical.

A comparison of the growth of litter mates receiving only vitamin B_1 or B_1 and hepatoflavin indicates the greater severity of conditions induced when the low fat, high sucrose diet is fed.

From the results of experiments with dialysis we conclude that both the filtrate factor and vitamin B_6 dialyze. Vitamin B_6 is removed from the dialysate by adsorption, but the activity is not evident from tests on the adsorbate. After 4 weeks of feeding we treated the remaining adsorbate with $Ba(OH)_2$, according to the method described for whole wheat concentrates, and fed the eluate. The surviving rats showed definite improvement in their condition and a gain in weight when the change was made. It seems probable that owing to gastric and intestinal disturbances the animals could not utilize the vitamin in the form of an adsorbate. The non-dialyzable material appeared to have no activity as regards either vitamin B_6 or the filtrate factor.

We were able to recover less than 25 per cent of the vitamin B_6 and filtrate factor activity from whole wheat by alcohol extraction. Apparently our method of adsorption and elution is efficient as regards vitamin B_6, since the eluate gave results closely similar to those of the solution. Also it seems that by this method there was a good separation of vitamin B_6 and the filtrate factor. The rats receiving the eluate were nearly protected from dermatitis but were losing weight rapidly when the filtrate was given in addition. The filtrate showed no vitamin B_6 potency, but it checked the weight loss and induced some gain in weight.

When rats received flavin alone or with 6 chick units of the repeatedly adsorbed liver extract, with or without the whole wheat filtrate, they developed dermatitis of about the same degree of severity. The liver preparation induced a gain of about 0.5 gm. per day. When the whole wheat eluate was fed with this liver filtrate, the rats were almost completely protected from dermatitis and the growth rate was improved. The whole wheat filtrate is not as rich in the filtrate factor as is the liver preparation.

The rice bran filtrate which had been subjected to two adsorptions is still rich in both vitamin B_6 and the filtrate factor. With

this material, however, two further adsorptions did not remove all of the vitamin B_6, since rats receiving the second filtrate developed only mild dermatitis.

SUMMARY

By the use of a scoring system, the relative potency of materials, as regards vitamin B_6, can be ascertained when account is taken of the degree of dermatitis developed in rats receiving only vitamin B_1 or B_1 and flavin.

Induced growth without protection from dermatitis apparently indicates the presence of the filtrate factor separate from vitamin B_6.

Vitamin B_6 was adsorbed and eluted with $Ba(OH)_2$ with almost complete recovery from liver and whole wheat preparations. Apparently four adsorptions of a rice bran extract were not sufficient to remove the vitamin B_6 activity completely. The liver extract prepared according to the method of Lepkovsky and Jukes contained no vitamin B_6 but was a good source of the filtrate factor.

Autoclaving at pH 9 for 1 hour does not appreciably destroy vitamin B_6 in liver filtrate, although its growth-promoting activity is lowered.

Both vitamin B_6 and the filtrate factor dialyze.

Vitamin B_6 activity in a liver filtrate was lost on storage in the cold, although the material was protected from light and bacterial action.

We did not find vitamin B_6 activity in wheat germ oil, nor destruction of any factor present in a brewers' yeast extract as a result of 10 hours irradiation.

From our experiments it would seem that an eluate from dialyzed freshly prepared liver filtrate or from whole wheat extract would be a promising source material to be used for further work in concentrating vitamin B_6.

Our experiments independently confirm the conclusions of Lepkovsky and Jukes in regard to the growth-stimulating action of the filtrate factor. Fractions prepared from whole wheat and from liver stimulated growth without protecting the rats from dermatitis.

Addendum—The results obtained with wheat germ oil and with irradiated brewers' yeast extract are at variance with those reported by Hogan and coworkers. In an attempt to determine the cause of the difference, we obtained from Professor Hogan supplies of his basal ration as well as of irradiated yeast extract, wheat germ oil, and tikitiki extract. When we used his technique with rats in this laboratory, however, our results still did not check. We then obtained three litters of young with their mothers from Dr. Hogan. During the first few weeks these rats responded as reported by Dr. Hogan and coworkers. Later, however, with the exception of one rat, they died rather rapidly regardless of additional supplement. This one animal was cured by wheat germ oil and gained in weight when flavin was supplied in addition. Animals which we sent to Hogan and Richardson conformed in general to Dr. Hogan's reported results when they were tested in the Missouri laboratory.

Such results can only be interpreted, we feel, as due to biological and climatic variations. The stock diets in the two laboratories are different, and the young rats undoubtedly carry different body stores of the various factors at weaning. The laboratories at the University of Missouri are maintained at a temperature of about 23.9–26.6°, while our laboratories are held at about 18.3–21.1°. Since the dermatitis syndromes as developed in the two laboratories appear to be identical, it would seem that differences we obtained when our rats received the Hogan diet and supplements were due to biological stores, whereas his animals were adversely affected by the temperature in our laboratory.

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THE ULTRAVIOLET ABSORPTION SPECTRUM OF CRYSTALLINE TOBACCO MOSAIC VIRUS PROTEIN

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A crystalline protein possessing the properties of tobacco mosaic virus has been isolated from mosaic-diseased Turkish tobacco plants (1). The evidence available at present indicates that this protein is the virus (1, 2); hence considerable interest is attached to studies of the properties of this active material. The ultraviolet absorption spectrum of tobacco mosaic virus protein has been determined and is presented in this paper.

Tobacco mosaic virus in juice from diseased plants or in purified or semipurified preparations has been found to become inactivated on exposure to ultraviolet light (3). Treatment of the crystalline virus protein with ultraviolet light has been found to result in the production of inactive, native protein retaining certain chemical and serological properties characteristic of the virus protein (4). The destruction spectrum of virus in a purified preparation has been determined and found to have a maximum at about 2650 Å. (5). The ultraviolet absorption spectrum of tobacco mosaic virus protein was found to be somewhat similar to that of other proteins, except that it is shifted towards the shorter wave-lengths and has an absorption maximum at 2650 Å. The previously determined destruction spectrum of the virus agent agrees essentially, therefore, with the absorption spectrum of the virus protein.

Investigation of the absorption spectrum of proteins containing the aromatic amino acids tryptophane, tyrosine, and phenylalanine has shown the presence of a band in the ultraviolet with the maximum of absorption at about 2800 Å. (6). It has also been found that under suitable experimental conditions the broad absorption band of proteins can be shown to consist of a number of narrow

bands which can be interpreted in terms of the constituent aromatic amino acids (7).   In the case of the virus protein, the band structure was found to differ in certain respects from that of other proteins.

<div align="center">EXPERIMENTAL</div>

The extinction coefficients were determined with a Spekker spectrophotometer and a small Hilger quartz spectrograph.   In order to locate the narrow bands it was found necessary to use a continuous light source.   These bands are faint and diffuse, and thus far it has not been possible to bring them out satisfactorily by use of a photometer and line source.   In the present experiments a hydrogen discharge tube and the small quartz spectrograph were used to obtain the bands.   All the solutions were photographed at room temperature in a phosphate buffer at pH 7.0.

<div align="center">

Results

</div>

The extinction coefficient curve of the virus protein, plotted against the wave-length in Ångström units, is shown in Fig. 1. The extinction coefficient is defined by the expression $\epsilon = 1/cl \log I_0/I$, where c is the concentration in moles per liter (1.2×10^{-8}), l is the cell length (2 cm.), I_0 is the intensity of light falling on the cell, and I is the intensity of light after passing through the thickness l.   The extinction coefficients were calculated on the basis of a molecular weight of 17 millions for the virus protein (8).

Also plotted in Fig. 1 is the extinction coefficient curve of pepsin. It can be seen that there is a marked difference in the position of the absorption maximum.   The curve for pepsin, as far as the positions of maxima and minima are concerned, is typical of the animal proteins which have been measured in this laboratory and agrees with the results of other investigators (6).

Little work, apparently, has been done upon the absorption spectra of plant proteins.   Measurements made on edestin (9) show that the maximum of absorption is at about 2800 Å.   We have checked this result and have determined the absorption curve of excelsin and find that this protein also has its maximum at 2800 Å.   The absorption spectrum of normal tobacco juice has a maximum at about 2650 Å., but the structure of this band is quite different from that of the virus protein.

If the absorption spectrum of the virus protein is obtained with a continuous light source, it can be shown that the broad band is made up of a number of narrow bands. Unfortunately, it has
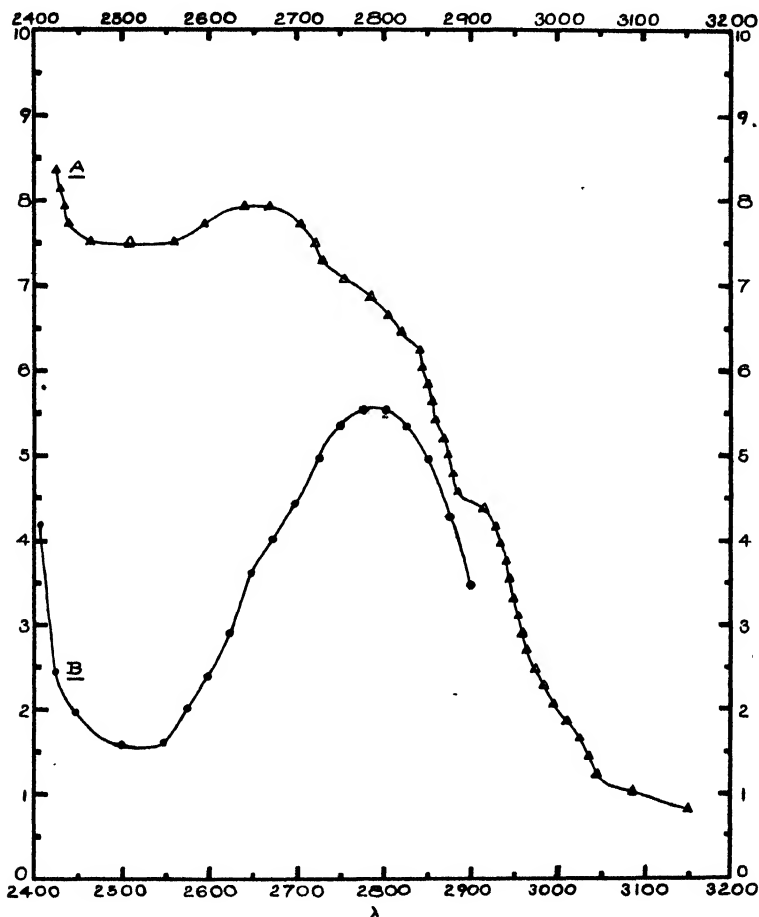
FIG. 1. Absorption spectrum of crystalline tobacco mosaic virus protein (Curve A) and of pepsin (Curve B). $\cdot \epsilon$ is to be multiplied by 10^7 for the virus protein and by 10^4 for pepsin.

been found difficult to reproduce these spectra photographically, so they have been read from the plates and plotted in Fig. 2. This method of illustration gives no idea of the relative intensity of the

bands, and at present an attempt is being made to devise a photometer with which to make this measurement. The bands are diffuse and it is quite possible that all of the structure has not been resolved.

The present interpretation of the narrow bands of pepsin is that the band at 2920 Å. is due to tryptophane, the one at 2840 Å. is due to tyrosine, the broad region from 2720 Å. to 2810 Å. is the result of overlapping of tryptophane and tyrosine, and those in the region of 2500 Å. to 2700 Å. are to be attributed to phenylalanine. The virus protein has the tryptophane band, and it appears to be in the same position as in the case of other proteins. Instead of the tyrosine band, there is a much wider band, shifted towards the ultraviolet, which has not been found in any of the
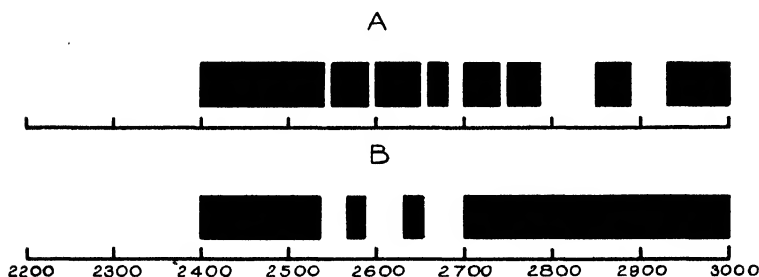
FIG. 2. *A* represents crystalline tobacco mosaic virus protein; *B*, normal tobacco juice. The scale is in Ångström units.

other proteins examined. No marked difference in the phenylalanine bands has been found. The tryptophane band is the sharpest and the last to disappear on dilution. The "modified tyrosine" band is quite distinct, but the bands in the phenylalanine region are still somewhat diffuse.

Some preliminary work has been done in an attempt to detect the virus protein in infectious tobacco juice by means of its characteristic absorption spectrum, and it was found that this could be done provided the juice had been subjected to a partial purification. The untreated juice from normal Turkish tobacco plants does not show the protein-like spectrum, but has a band as illustrated in Fig. 2. Since this band disappears on removal of the pigment, we shall refer to it as the pigment band. Along with this

band there is also a trace of the tryptophane band. The untreated juice from mosaic-diseased Turkish tobacco plants of the same age has this pigment band and a good tryptophane band. The modified tyrosine band which is taken to be characteristic of the virus protein was not detected, so it is not known whether this increase in absorption in the tryptophane region is due to the virus protein or to some other globulin.

In order to determine the effect of removing the pigment, the globulin fraction of both of the juices was precipitated with ammonium sulfate, removed by filtration, and dissolved in 0.1 M phosphate buffer at pH 7.0. The absorption spectrum of the normal juice showed that the pigment band was still present and that the tryptophane band was more distinct. The infected juice now showed the presence of the modified tyrosine band and the pigment band was just discernible. Further purification caused the pigment band to disappear. In the case of the normal juice, the final state was a hazy absorption in the region of 2600 Å. to 2700 Å. and the tryptophane band. The absorption spectrum of the crystalline virus protein is shown in Fig. 2.

SUMMARY

The ultraviolet absorption spectrum of crystalline tobacco mosaic virus protein has been determined and found to agree essentially with the destruction spectrum previously found for the virus agent in purified preparations. The virus protein has an absorption band in the ultraviolet with the absorption maximum at 2650 Å. The structure of the band is different from that of other proteins, for the band in the region of tyrosine absorption has been shifted towards the ultraviolet. It has been possible to demonstrate the presence of the virus protein in the partially purified juice from mosaic-diseased Turkish tobacco plants by means of ultraviolet absorption spectrum measurements.

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THE DISTRIBUTION OF BASES BETWEEN CELLS AND SERUM OF NORMAL HUMAN BLOOD*

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A renewed attempt has been made to determine the distribution of inorganic bases between cells and serum of normal human blood. The investigation was undertaken, first, for its descriptive value; second, because blood affords an opportunity to compare cells with their native interstitial fluid; third, to provide a basis for the study of the reactions of these cells to changes of environment both in the test-tube and in the body.

The composition of red blood cells has received frequent attention since Schmidt's (1) classical analyses. However, all examinations have been open to criticism for one or more reasons. Schmidt and numerous subsequent observers treated blood as if it were an entirely indifferent fluid, paying no attention to the fact that it has a vitality of its own that is expressed even in the test-tube by metabolic activities of a complex nature. Moreover, Schmidt, having no satisfactory means of separating cells from serum, was forced to rely upon spontaneous sedimentation. Since then numerous devices have been employed, but few investigators have controlled their methods for the separation or measurement of the cells with the same care which many have given to more purely analytical procedures.

The expedients employed to permit the allocation of solutes to cells and serum involve one of three principles. First, cells packed by centrifugation have been sampled directly. Hamburger (2) early demonstrated that the volume which cells attained in a centrifuge tube was significantly larger than the volume to which

they could be packed in fine tubes. This indicated, although it did not prove, that a certain amount of serum remained between the cells. To remove this residual serum some workers have resorted to washing the cells with various fluids, usually isotonic glucose solutions. Glucose, however, can penetrate red blood cells and is utilized in these cells for certain metabolic processes. There can be no assurance that washing with glucose is without effect on the internal composition of red blood cells.

The only valid procedure appears to lie in analysis of whole blood and serum with separate measurement of the volume of the red blood cells. Hamburger (2) found that if cells were packed in fine tubes by centrifugation until they reached a constant volume, they became transparent, indicating that the serum had been completely expressed from their interstices. He believed, however, that satisfactory separation could be effected only if blood were diluted. Others, among them the authors, have found that the criteria of separation, established by Hamburger, can be secured with undiluted blood in capillary tubes. Certain earlier work on the permeability of the red blood cell membrane to various solutes (3) convinced us that the hematocrit, if properly employed, afforded accurate measurements of changes in the volume of the red blood cells. This led to further studies, which have been recently published (4), in which the absolute accuracy of the method was established by measurements of the changes in concentration of solutes in serum caused by diluting the blood with isotonic sucrose.

Meanwhile, methods for the analysis of blood were carefully examined and improved until a procedure has been developed which permits the accurate determination of calcium, magnesium, sodium, potassium, the combined sulfates of sodium and potassium, and the total base of serum or blood, with great economy of material (5).

Methods

The subjects of the investigation were twenty normal adults, physicians, laboratory workers, and medical students. In all twenty-two specimens of blood were examined. The subjects were in the postabsorptive state. Venous blood was withdrawn without stasis and treated anaerobically throughout.

One portion was transferred at once to a mercury sampling bulb in which it was defibrinated by the anaerobic technique of Eisenman (6). This was used for measurements of cell volume by the method of Eisenman (4), carbon monoxide capacity by the method of Van Slyke and Hiller (7), water, by weighing 2 cc. aliquots before and after drying to constant weight in Pyrex dishes in an electric oven at 93–97°, bases by the method of Hald (5) with modifications which will be described below, carbon dioxide by the method of Van Slyke and Neill (8), chloride by Eisenman's modification of the method of Van Slyke (9) or Hald's modification of Patterson's microtechnique (10).

A second portion of blood was transferred to a centrifuge tube under paraffin oil. After the blood had clotted, the tube, capped by a rubber stopper which displaced the oil, was centrifuged. The serum was transferred anaerobically to a sampling bulb over mercury, from which aliquots were removed for determination of carbon dioxide, chloride, water, and bases by methods similar to those employed for the analysis of blood. In addition total protein was estimated from determinations of total and non-protein nitrogen.

In most instances, besides the individual bases, Ca, Mg, K, and Na, and total base, the combined sulfates of Na + K were determined. This provided a means of checking the accuracy of the estimations of sodium and potassium, and in addition afforded three more or less independent measurements of total base as, (1) [Ca] + [Mg] + [K] + [Na], (2) [Ca] + [Mg] + ([K + Na] as combined sulfates), (3) total base, directly determined.

Total base was determined by the gravimetric benzidine technique in all instances.

Enough material was obtained, in the last seventeen studies, to make it possible for potassium and sodium to be determined directly on an independent ash instead of on the filtrate which remained after calcium and magnesium had been removed. Instead of ashing a single cc. for total base, portions of 2 cc. were used, phosphate being removed in the usual manner. The phosphorus-free filtrate was divided into two portions, one of which was used for potassium followed by sodium, the other for total base. By using this procedure, a study could be completed in much less time. No difference in the accuracy of the results was apparent.

In ashing theoretical solutions of the various salts it has been observed that many of them creep during the dry ashing, giving low results when this occurs. A small amount of sucrose added to the solution furnishes an organic mass which seems to prevent creeping, yielding an ash which forms and remains in the bottom of the dish.

The concentrations of the various solutes and water in the red blood cells were calculated by the following equation

$$\frac{S_b - S_s V_s}{V_c}$$

in which S = the concentration of the solute in question, V = volume, and the subscripts, b, s, and c, represent, respectively, blood, serum, and cells.

Results

The analytical data from sixteen studies of sixteen individuals are presented in Table I. The first six studies have been omitted because there was reason to believe that magnesium in whole blood was not completely recovered. With the exception of the figures for magnesium the studies which were omitted are in essential agreement with the sixteen in Table I.

Calcium of serum is of the generally accepted magnitude; just how much there may be in the cells it is hard to say. In two instances, M.H. and G.K., there appear to be significant quantities; in most bloods, however, there are only equivocal amounts, so small that estimations are of no value. Although it is impossible to say that calcium is excluded from cells entirely, the data confirm previous opinions (11–15) that it is an insignificant constituent of cells. The concentration of magnesium, on the other hand, is greater in cells than in serum, as Becher (16) and Greenberg *et al.* (17) also found. Potassium, as Schmidt (1) and most subsequent observers (11, 18–20) have found, is the predominant inorganic base of cells. The red blood cell is not entirely free from sodium, as Kramer and Tisdall (11) suggested.

Before the concentrations of sodium and potassium are discussed in detail, however, it is necessary to consider certain questions which these data raise. The concentrations of sodium in serum are, as Hald (5) has already pointed out, lower than the

values which are usually given (1, 21–31).   The same is true of
the concentrations of total base.   Butler and MacKay (29), for

TABLE I

Concentrations of Bases and Water in Serum and Whole Blood;
Analytical Data

Subject		M.-eq. per liter						Per cent	
		Ca	Mg	K	Na	Combined SO₄	B	H₂O	Volume
M. H.	Serum	5.3	1.2	3.9	134.3	138.4	144.4	93.9	56.4
	Blood	3.3	2.6	41.9	81.6	123.2	130.6	71 8	43.6
C. W.	Serum	5.1	2.0	4.1	135.1	141.1	147.2	93.6	55.7
	Blood	3.0	3.0	40.7	83.5	122.8	129.5	69.9	44.3
C. R.	Serum	5.6	1.3	4.0	137.1	140.6	147.5	93.3	52.4
	Blood	2.9	2.6	42.1	80.0	119.4	127.7	71.3	47.6
H. R.	Serum	5.5	1.6	4.1	136.3	141.8	146.9	92.4	52.3
	Blood	2.9	2.7	36.4	78.5	113.8	121.1	69.9	47.7
A. D.	Serum	5.0	1 3	4.9	135.8	140.5	148.7	94.4	58.4
	Blood	2.9	2.3	36·6	90.6	127.8	131.4	69.2	41.6
A. G.	Serum	5.0	1.6	5.4	135.6		147.4	93.3	56.3
	Blood	2.8	2.4	37.3	86.6	124.9	128.8	69.2	43.7
E. S.	Serum	4.8	1.4	4.5	129.2	134.3	139.0	94.0	53.3
	Blood	2.5	2.9	41.8	77.3	120.5	125.3	69.4	46.7
K.	Serum	5.7	1.6	3.8	138.7	143.1	149.4	94.2	56.3
	Blood	3.4	2.8	37.8	85.0	121.9	129.6	70.2	43.7
D. L.	Serum	5.6	1.4	3.9	138.9	143.6	150.0	94.0	56.9
	Blood	3.1	3.2	37.1	84.6	121.5	127.7	71.8	43.1
M.	Serum	5.1	1.5	4.3	134.5	139.7	145.8	94.2	57.6
	Blood	3.0	2.9	35.4	84.4	120.2	125.4	70.5	42.4
A. W.	Serum	5.1	1.2	4.6	134.7		146.1	93.4	56.4
	Blood	3.1	2.7	40.0	80.3		126.6	71.6	43.6
A. C.	Serum	5.8	1.8	3.4	134.8	138.7	144.9	93.9	59.2
	Blood	3.5	2.9	35.7	87.3	123.1	129.3	71.5	40.8
A. P.	Serum	5.7	2.2	4.3	134.0		146.8	93.9	53.4
	Blood	3.2	4.0	49.6	76.2		133.5	71.9	46.6
G. K.	Serum	5.6	2 0	5 9	136.0	142.4	150.9	93.4	54.2
	Blood	3.7	3.5	39.7	83.4	123.4	131.2	72.0	45.8
J. P.	Serum	5.4	1.7	6.5	134.3	139.8	149.0	93.7	60.3
	Blood	3.1	2.9	33.9	85.8	120.3	133.1	70.8	39.7
P. H.	Serum	5.0	2.1	5.2	129.8	136.3	144.0	93.6	57.6·
	Blood	2.7	3.4	34.5	83.2	119.0	124.3	72.5	42.4

instance, found 141 to 145 milli-equivalents of sodium and 155
to 160 of base.   In a recent paper Gutman *et al.* (32) report about

139 to 142 milli-equivalents of Na and 150 to 153 of base in normal individuals. Keys (30) found 138 to 154 milli-equivalents of Na and 150 to 169 of base. It must be confessed that earlier estimates of total base from this laboratory (33, 34) were equally high. It is believed that our own high values were referable to certain technical errors and that those already published by Hald (5) and these in Table I are more nearly correct.

There are numerous reasons for this opinion. Perhaps the most cogent, although the least direct, lies in the excellent agreement of the values for total base secured by three methods: the sum of the individual bases; the sum of calcium, magnesium, and the combined sulfates of sodium and potassium; and the direct gravimetric determination of total base. In the thirteen experiments in which all three methods were used, the difference between the two furthest apart averaged only 1.4 milli-equivalents and never exceeded 2.3 milli-equivalents. In all sixteen experiments (in three combined sulfates were not determined), the difference between the two methods which were in closest agreement averaged 0.4 and never exceeded 1.0 milli-equivalent. Furthermore, there was no systematic tendency for one method to yield higher values than another. Total base was lowest seven times, intermediate six times; the sum of the bases lowest seven times, intermediate eight (in one blood total base and base from combined sulfates were identical and lower than the sum of the bases). Such agreement could hardly be obtained if the methods were not based on sound principles. The only step which is common to all is the preliminary ashing in a muffle furnace. It has been established by extensive control experiments that no base is lost in this process. In order to make assurance doubly sure a known solution of salts was usually run through the analytical procedure, including ashing, with each blood. In addition the same amounts of total base and of individual bases are recovered if serum or blood is prepared by wet ashing instead of muffling.

The analysis of whole blood, judging by recovery experiments and by differences between duplicates, is quite as accurate as the analysis of serum. The estimation of concentrations in cells is, however, subject to a further error of calculation. If the mean error in the determination of base or sodium in serum or in whole blood is $\pm$ 1.0 milli-equivalent, a conjunction of errors could

give a total error of 2.0 milli-equivalents. The measurement of cell volume has a possible error of 1 volume per cent. If this were added to the analytical errors and all fell in the same direction, the estimated concentration of base or sodium in the cells might be faulty by as much as 4.0 milli-equivalents. That the actual error can seldom exceed this estimation is witnessed by certain experiments presented in another connection ((30) Table IV). In these experiments both the volumes of the cells and the concentrations of base in blood and serum were greatly altered by the addition to blood of water and various salts. In no case were more than 4 milli-equivalents of base found to have crossed the cell membranes. Moreover, separate calculations from analyses of serum alone and of whole blood and serum together agreed in the direction of these apparent transfers. Such agreement would have been impossible if the errors of analysis or measurements of cell volume had exceeded the limits defined above.

To reduce errors further advantage has been taken of the fact that base was determined by three methods. In Table II are given the most plausible values for the bases of serum and cells. These were selected in the following manner. If the three values for total base agreed within 2 milli-equivalents, they were averaged. When the spread was greater than 2 milli-equivalents and two of the three values were close together, these two were averaged (this was true of only one serum and two bloods); otherwise an average of the three values was used. When the corrected value for total base did not agree with the sum of the individual bases, it was necessary to bring the latter into conformity. In serum, since the errors in measurement of Ca, Mg, and K were negligible in comparison to the error of measuring Na, the correction was applied entirely to Na. In whole blood the correction was divided between Na and K in proportion to their relative magnitudes. The corrections were usually of no great importance; when they did attain significance, they seemed to reduce the variability in concentrations and distributions of Na + K slightly, but appreciably.

Except for the low values of Na and total base, the values for inorganic cations in serum agree with those reported by other observers. The range of variation of all the basic components is relatively small. This is not true of the same constituents in cells.

If there is any calcium at all in the cells, it forms a negligible pro-
portion of the total base. Magnesium makes up the major

TABLE II

Concentrations of Bases in Serum and Cells, Derived from Data of Table I

Subject		Corrected m.-eq. per liter						M.-eq. per kilo water			
		Ca	Mg	K	Na	$\frac{Na+}{K}$	B	$\frac{Na+}{K}$	B	$\frac{(Na+K)_c}{(Na+K)_s}$	$\frac{B_c}{B_s}$
M. H.	Serum	5.3	1.2	3.9	134.3	138.2	144.7	147.1	154.0	0.994	0.998
	Cells	0.8	4.4	91.3	13.8	105.1	110.3	146.2	153.5		
C. W.	Serum	5.1	2.0	4.1	135.8	139.9	147.0	149.4	156.9	0.985	0.979
	Cells	0.4	4.1	86.0	16.9	102.9	107.4	147.1	153.5		
C. R.	Serum	5.6	1.3	4.0	136.8	140.8	147.7	150.8	158.2	0.943	0.935
	Cells	0	4.1	83.9	17.5	101.4	105.5	142.2	147.8		
H. R.	Serum	5.5	1.6	4.1	136.0	140.1	147.2	151.6	159.2	0.822	0.929
	Cells	0.1	3.9	71.8	15.4	87.2	91.2	124.7	131.8		
A. D.	Serum	5.0	1.3	4.9	135.7	140.6	146.9	148.8	155.5	1.049	1.038
	Cells	−0.1	3.7	81.1	27.1	108.2	111.9	156.1	161.5		
A. G.	Serum	5.0	1.6	5.4	135.5	140.9	147.5	150.9	157.9	0.982	0.969
	Cells	−0.2	3.5	78.7	23.9	102.6	105.9	148.2	153.0		
E. S.	Serum	4.8	1.4	4.5	129.1	133.6	139.8	142.0	149.8	1.055	1.042
	Cells	−0.1	4.6	84.9	19.1	104.0	108.5	149.8	156.2		
K.	Serum	5.7	1.6	3.8	138.8	142.6	149.9	151.3	159.0	0.914	0.912
	Cells	0.4	4.3	81.7	15.4	97.1	101.8	138.2	145.0		
D. L.	Serum	5.6	1.4	3.9	139.2	143.1	150.1	152.2	159.6	0.850	0.859
	Cells	−0.2	5.7	80.7	12.3	93.0	98.5	129.4	137.0		
M.	Serum	5.1	1.5	4.4	134.8	139.2	145.8	147.7	154.7	0.896	0.901
	Cells	0.2	4.7	77.6	15.8	93.4	98.3	132.3	139.3		
A. W.	Serum	5.1	1.2	4.6	135.0	139.6	145.9	149.5	156.2	0.895	0.903
	Cells	0.5	4.6	85.9	10.0	95.9	101.0	133.8	141.0		
A. C.	Serum	5.8	1.8	3.4	134.7	138.1	145.5	147.0	154.8	0.923	0.919
	Cells	0.3	4.5	82.7	18.4	101.1	105.9	135.6	142.1		
A. P.	Serum	5.7	2.2	4.3	134.3	138.6	146.5	147.5	156.0	1.051	1.051
	Cells	0.3	6.2	101.7	10.0	111.7	118.2	155.2	164.3		
G. K.	Serum	5.6	2.0	6.0	136.5	142.5	150.1	152.5	160.6	0.919	0.930
	Cells	1.4	5.3	79.9	21.0	100.9	107.6	140.1	149.3		
J. P.	Serum	5.4	1.7	6.5	134.3	140.8	147.9	150.1	157.6	0.933	0.931
	Cells	−0.5	4.6	75.8	12.8	88.6	92.7	125.0	130.8		
P. H.	Serum	5.0	2.1	5.4	130.7	136.1	143.2	145.3	152.9	0.892	0.892
	Cells	−0.3	5.1	74.7	19.4	94.1	98.9	129.6	136.3		

portion of the bivalent base in blood cells as it seems to do in most
tissue cells. Both together contribute only a small fraction of
the total base, which is composed chiefly of sodium and potas-

sium.[1] Although the latter predominates, sodium plays a far from negligible rôle. The range of variation of both sodium and potassium is large. The literature on the subject yields nothing but confusion. The values for sodium range from -7 to $+3$ (11) to 20 to 71 milli-equivalents per liter (1). With the exception of Kramer and Tisdall (11), however, all observers (1, 26, 29, 35) agree that sodium appears in appreciable quantities in human red blood cells. The present figures fall within the average range of those in the literature.

While Wanach (26) found only 7 to 8 milli-equivalents of K per liter of cells, Remond and Cantegril (20) report 155 to 161. Intermediate concentrations, found by other analysts (1, 17–19) usually vary from 80 to 120 milli-equivalents, agreeing in general magnitude with those here reported.

Table III shows the range of variation of concentrations of base in cells and serum. The greater variability of the cellular constituents is at once evident. Potassium varies more in absolute magnitude than any other component, but, in relation to its concentration, the variation of sodium is far greater. The variations of both appear to exceed greatly the errors of the analytical methods. Although the sum, Na + K, varies less than the sum of the variations of Na and K, it varies quite as much as K, both in absolute figures and in per cent. There is, therefore, no definite reciprocal relation between the two. Moreover, total base is no more constant than Na + K. This is to be expected since Mg forms such a small and relatively constant fraction of base.

In the last four columns of Table II and the second half of Table III the concentrations of Na + K and of B are given in relation to the water of cells and serum. From the standpoint of osmotic equilibrium, in which electrolytes play so large a part, these values should have more significance than those relating concentration to volume and might be expected to be more constant. Actually they are quite as variable. It can be inferred that there is no close correlation between the concentrations of base and of protein, which makes up the major portion of the solids of the cells. Finally, the ratios $(Na + K)_c/(Na + K)_s$ and

[1] Estimated as milli-equivalents per kilo of water, the concentrations of bivalent base, Ca + Mg, in serum and cells are of approximately the same magnitude.

B_c/B_s (in terms of milli-equivalents per kilo of water) have the same degree of variation as the functions $(Na + K)_c$ and B_c. It follows that the concentrations of base in the cells are not directly related to those in the serum.

For comparison the literature offers only the scantiest data on the concentration of total base in normal human red blood cells.

TABLE III

Variability of Base in Serum and Cells

	Serum					Cells				
	Concentrations			Average deviation		Concentrations			Average deviation	
	M.-eq. per liter				*per cent*	M.-eq. per liter				*per cent*
	Maximum	Minimum	Average			Maximum	Minimum	Average		
Ca	5.8	4.8	5.3	0.3	6	1.4	−0.5	0.2	0.3	150
Mg	2.2	1.2	1.6	0.3	16	6.2	3.5	4.6	0.5	11
K	6.5	3.4	4.6	0.7	14	101.7	71.8	82.5	4.9	6
Na	139.2	129.1	135.1	1.7	1	27.1	10.0	16.8	3.5	21
" + K	142.6	133.6	139.7	1.8	1	111.7	87.2	99.2	5.5	6
B	150.1	139.8	146.6	1.9	1	118.2	91.2	104.0	5.7	6
	M.-eq. per kilo water					M.-eq. per kilo water				
Na + K	152.5	142.0	149.0	2.2	1	156.1	124.7	139.6	8.3	6
B	160 6	149.8	156.4	2.1	1	164 3	130.8	146.4	7.9	5

	Maximum	Minimum	Average	Average deviation
				per cent
$\dfrac{(Na + K)_c}{(Na + K)_s}$ per kilo of water......	1.055	0.822	0.944	6
$\dfrac{B_c}{B_s}$ per kilo of water...............	1.051	0.859	0.949	5

Schmidt (1) gives figures from two subjects; but his methods for the separation of cells were quite inadequate. Kramer and Tisdall (11) offer certain data from which rough estimations have been made (31). However, they did not analyze cells and serum of the same bloods for all basic constituents. Figures derived from the blood of other animals cannot be used because of the known variability of species. Butler and MacKay (29) analyzed

both cells and serum of two normal individuals (the authors themselves) for sodium, potassium, and total base. Their values for sodium of cells, 15 to 23 milli-equivalents (average 18), are similar in magnitude to our own, although they found more sodium in serum. Their potassium figures for cells, 52 to 55 milli-equivalents (average 54), are lower than those reported in this paper or by most other reliable observers, while their figures for total base in both cells and serum are higher than ours. It seems quite evident that Butler and MacKay have made some consistent analytical error, because differences between B and Na + K in their data are far larger than any reported values·for Ca + Mg in cells.

DISCUSSION

There is reason to believe that the osmotic pressure of the contents of the red blood cells is essentially the same as that of the serum and that the cells respond as osmometers to changes in the composition of the serum. The envelope of the resting[2] red blood cell appears to be freely permeable to water, organic substances of small molecular size, the hydrogen ion, ammonia, chloride, and bicarbonate, but impermeable to proteins, lipids, sucrose, and other organic molecules of large size, to inorganic cations, to phosphate (36), and to sulfate. It follows that the concentration of inorganic cations must be an important determinant of the osmotic pressure of the cellular contents. Variations in the concentration of these cations must alter osmotic equilibrium, inducing compensatory transfers of water between the two phases of the blood.

It is somewhat disconcerting, then, to find the concentration of inorganic base in the cells so variable without clear relation to the concentration of base in serum or to the proportion of water or protein in the cells. In the face of such inconstancy it is idle to attempt to treat the data on rigid thermodynamic principles. That such principles govern osmotic equilibria between cells and serum can be assumed, even if it were not attested by experiments

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[2] The term resting is used to describe the condition in which metabolic and autolytic processes, such as glycolysis, are held in abeyance or retarded. It has been demonstrated recently (36) that phosphate and potassium traverse the red blood cell membrane when cellular metabolism is active.

in which the equilibria have been disturbed by alteration of separate variables. As a static system, however, blood contains so many variables and unknown factors that it defies analysis. One can hardly go further than to list some of the most obvious of these difficulties. No precedent can be found for the treatment of a system in which both inorganic cations and certain anions are restrained. There is no satisfactory explanation for the disparate distribution of potassium and sodium. The occurrence in the cells of both ions in variable proportions only adds to the mystery. The raw concentrations of electrolytes can have but little significance from the standpoint of osmotic pressure. The activities of the ions should be known, but cannot even be reasonably conjectured. There is some question whether all the water in the cells is available for solvent purposes.

In another connection (37) the averages of the present data were treated at some length without consideration of the variability of the figures from which they were derived. However, such treatment is of value only as a means of stating the problem. No general deductions can be drawn from averages unless the deviations from the average can be ascribed to errors. The range of magnitude of the values for total base does, perhaps, warrant some attention. Van Slyke, Wu, and McLean (38), in their study of electrolyte equilibria in the blood of a Manchurian pony, found that the concentration of base per unit of water in cells exceeded that in serum. This was true in only three of the sixteen samples of blood in the present study. If the assumption is made that the base is all ionized and active and if the cell membranes are impermeable to both base and protein, osmotic equality on the two sides of the membrane could be secured only if base were, as it is in most of our experiments, more concentrated in the medium containing less protein, the serum (39). Since presumably a larger proportion of the base in cells is combined with protein in undissociated form, the concentration of active inorganic cations may be lower in the serum even in those instances in which the distribution coefficients estimated from total concentrations of base or $Na + K$ are greater than 1.

SUMMARY

The concentrations of water and inorganic bases in the serum and red blood cells of sixteen normal adults have been estimated

from analyses of serum and whole blood and measurements of cell volume.

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OSMOTIC ADJUSTMENTS BETWEEN CELLS AND SERUM IN THE CIRCULATING BLOOD OF MAN*

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It has been demonstrated previously that the erythrocytes of well preserved blood are impermeable to the basic elements Na and K (1). Adjustments of osmotic pressure after the addition to blood of water or of salts of sodium or potassium are made entirely by transfers of water between cells and serum. With this point established, an attempt has been made to determine whether in the circulating blood base is as sharply segregated and whether osmotic adjustments are also made merely by transfers of water.

Procedure

Patients with severe dehydration and serum base deficiency were chosen as subjects. After a preliminary sample of blood had been taken for analysis, the patient was given by intravenous infusion enough hypertonic solution of sodium chloride or sodium sulfate to raise the serum base considerably. Shortly after the infusion was completed, a second sample of blood was withdrawn for analysis. In one instance another sample was taken after a subsequent interval. The bloods were taken at no especial time of day and with no particular relation to meals; but the patients were so ill that they had been unable for some time before the experiments to take or retain food and fluids. Therefore, the experiments represent conditions existing after various periods of starvation and thirst. Scant attention will be given to the clinical conditions of the patients, since these are, for the most part, irrelevant to the purposes of this discussion.

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Blood was withdrawn from an antecubital vein without stasis under anaerobic precautions. Part was defibrinated anaerobically over mercury. The remainder was transferred to centrifuge tubes under oil. After it had clotted, the oil was displaced by a rubber stopper, the blood centrifuged, and the serum removed to a sampling bulb over mercury. The defibrinated blood was used for measurement of cell volume and oxygen capacity and for determination of total base. The methods have been described in detail elsewhere (2). For total base the gravimetric method of Hald (3) was employed.

Calculations

When blood is diluted *in vivo* by the intravenous injection of fluid, evaluation of the results on the composition of cells and serum is more difficult than it is when blood is diluted in the test-tube, because the blood stream is not a self-contained system. Fluid may enter or leave the blood vessels; cells may be added to or withdrawn from the circulation. It is, therefore, necessary in calculations to use some method which will identify in the altered blood the units of cells and serum from the initial blood. As a means of identification hemoglobin (oxygen capacity) has been chosen.

The water in that volume of cells in the second blood which contains the same amount of hemoglobin as 100 cc. of the first blood is estimated by the following equation

$$W_{c_2} = W_{c_1} - 100 \left[1 - \frac{Hb_1}{} \right.$$

in which Hb = oxygen capacity of whole blood, V_c = cell volume, W_c = cell water, and the subscripts 1 and 2 represent respectively the first and second bloods.

Water of cells in the initial blood, W_{c_1}, was not determined directly, but was calculated by means of the equation $W_c = 94.53 - 0.704\, Hb_c$ derived by Eisenman, Mackenzie, and Peters (4). It will be noted that W_{c_2} was not estimated in the same manner, but was derived from W_{c_1}, with the assumption that changes of cell volume were entirely referable to transfers of water. This

assumption can introduce no appreciable error unless some solids of large molecular volume are transferred.   The non-hemoglobin solids in the cells are, however, too great to permit the use of the formula for the estimation of both W_{c_1} and W_{c_2}.

Water of serum, W_s, was estimated from serum proteins by the formula, $98.4 - 0.718\ P_s = W_s$, also derived by Eisenman, Mackenzie, and Peters (4).

The quantity of base, B'_{c_2}, in that volume of cells of the second blood which contains the same amount of hemoglobin as 100 cc. of the first blood is estimated by the following equation

$$\left(\ \frac{\dfrac{Hb_1}{V_{c_1}}}{\dfrac{Hb_2}{V_{c_2}}}\ B_{c_2}\ \right] = B'_{c_2}$$

in which B_c represents the concentration of base in a unit volume of cells.

Results

Analytical data are given in Table I; values derived by calculation from these data in Table II.   In every instance but one (Ti, 1–2) the injection of hypertonic salt solution, whether as sodium chloride or sodium sulfate, caused the red cells to contract, yielding water to serum.   The actual expansion of the serum, as estimated from the change of protein, was too great to be accounted for entirely by the water derived from the cells.   Either some of the injected fluid had not escaped from the blood stream, or some water had been drawn from the interstitial fluids.   In every case, even when blood was withdrawn within a few minutes of the end of the infusion (the introduction of fluid usually took the better part of an hour), most of the injected base and water had left the blood stream.   If it is assumed that the initial blood volume in every case was 5000 cc., the proportion of injected water exceeds the proportion of injected salt retained in the serum in M, Be, and Cr.   The two are approximately equal in Bo, Ch, and S, while the proportion of base retained is greater in Tr. (Ti is omitted from consideration for reasons that will be mentioned later.)   The total amounts of salt or water retained in the circulation by Bo, Ch, and Tr are so small as to be of doubtful

significance.   For the most part base would seem to diffuse more rapidly than water out of the blood stream.   This is to be expected, since the hypertonic serum would tend to draw water from the interstitial fluids, while base would diffuse outward into the tissues where its concentration was lower.

If the red blood cells act as perfect osmometers, with envelopes impermeable to base, the change of concentration of water within

TABLE I

Blood and Serum before and after Intravenous Injections of Salt Solution

Subject		Whole blood			Serum		Nature of infusion and duration of experiment*
		Cell volume	O₂ capacity	Base	Protein	Base	
		per cent	per cent	m.-eq.	per cent	m.-eq.	
M	Before	42.1	15.2	118.8	3.93	134.4	750 cc. 2% NaCl
	After	34.1	13.4	131.6	3.21	142.7	Immediately after
Ti	Before	54.0		134.8	8.13	127.9	1500 cc. 2.5% NaCl
	After	38.8	14.3	133.0	5.43	156.0	Immediately after
	Later	45.2	16.1	121.0	6.25	150.9	18 hrs. later
Bo	Before	22.0	9.0	126.5	5.15	134.4	1500 cc. 2% NaCl
	After	19.6	8.5	130.4	4.92	140.0	17.5 hrs. later
Be	Before	47.5	20.5	131.2	6.44	148.8	400 cc. H₂O, 240 m.-eq. Na₂SO₄
	After	41.8	18.6	135.2	5.62	154.5	Immediately after
Cr	Before	39.4	18.1	130.0	7.25	148.6	500 cc. H₂O, 266 m.-eq. Na₂SO₄
	After	34.7	16.7	143.2	6.54	155.9	Immediately after
Ch	Before	41.7	17.9	130.9	5.24	147.6	1000 cc. 2% NaCl
	After	39.3	17.5	135.4	4.90	153.9	Immediately after
S	Before	35.6	14.3	129.3	6.71	142.8	1000 cc. 2% NaCl
	After	29.8	12.5	135.3	6.10	151.3	Immediately after
Tr	Before	44.2	17.1	121.6	5.08	133.5	500 cc. 3% NaCl
	After	38.8	15.3	128.8	5.08	141.5	Immediately after

* "Immediately after" indicates that the second blood was withdrawn within 15 minutes of the end of the infusion.

the cells should be proportional to the change of concentration of base per unit of water in the serum; that is, W_{c_2}/W_{c_1} should equal B_{s_1}/B_{s_2}.   A glance at the last two columns of Table II will show that in every case but Ti (1–2 and 1–3) water of cells and base of serum have deviated in the same direction; the cells have contracted when base of serum increased.   It is suspected that the values for either oxygen capacity or cell volume in the first obser-

vation on Ti are erroneous.   This would explain the fact that the base of cells on this occasion exceeded the base of serum and also the fact that the cells appeared to shrink between the first two observations.   It will be seen that between the second and third (Ti, 2–3), as the base of the serum became diluted, the cells swelled according to expectation.   If the first observation on Ti is neglected, the magnitudes of the ratios of cell water and of serum base are in satisfactory agreement in every case except possibly for M.   The maximum total error of estimating the ratio W_{c_2}/W_{c_1} is probably about 5 per cent.   The discrepancy between

TABLE II

Changes of Water and Base in Blood Cells and Serum after Intravenous Injections of Salt Solution

Subject	Water of cells		Base of serum, per liter H₂O		Base of cells		Transfer of base to cells $B'_{c_2} - B_{c_1}$	$\dfrac{W_{c_2}}{W_{c_1}}$	$\dfrac{B_{s_1}}{B_{s_2}}$
	W_{c_1}	W_{c_2}	B_{s_1}	B_{s_2}	B_{c_1}	B'_{c_2}*			
	vol. per cent	*vol. per cent*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*		
M	75.6	67.5	140.6	148.5	97.4	101.3	3.9	89.3	94.7
Ti, 1–2	75.3	75.3	138.1	165 1	140.6	96.6	−44.0	100.0	83.6
1–3	75.3	72 8	138.1	160.7	140.6	87.0	−53.6	96.6	85.9
2–3	75.2	71.8	165.1	160.7	96.6	87.7	−8.9	104.7	102.7
Bo	73.0	68.1	141.9	147.5	98 6	86 3	−12.3	93.3	96.2
Be	71.9	69.0	158.6	163.6	111.7	105.1	−6.6	96.0	97.0
Cr	70.5	65.7	159.4	166.4	101.4	113.5	12.1	93.2	95.8
Ch	71.9	68.5	156.0	162.2	107.6	102.2	−5.4	95.3	96.2
S	73.5	69.2	152.6	161.0	104.9	93.4	−11.5	94.2	94.8
Tr	74.2	73.2	140.8	149.3	106.6	107.5	0.9	98.7	94.3

* B'_{c_2} represents the concentration of base in a unit of original cells.

W_{c_2}/W_{c_1} and B_{s_1}/B_{s_2}, even in M, is only 5.4 per cent.   In all other cases, with the exception of Ti, noted above, it is below 5 per cent, in all but one less than 3 per cent, and in three 1 per cent or less.   Considering the errors of experimental methods, then, the cells appear to act like osmometers with membranes that allow the passage of water, but no base.   It is doubtful whether any significance should be attached to the fact that W_{c_2}/W_{c_1} is lower than B_{s_1}/B_{s_2} six out of seven times when B_s increases, and higher on the only occasion (Ti, 2–3) when B decreases.

In contrast with this when the actual amounts of base in cells

before and after treatment, B_{c_1} and B'_{c_1}, are compared, base is found to have crossed the cell membrane in all but two instances (M and Tr). The error of estimating base transfers in these experiments should not exceed 4 per cent. Certainly it can hardly explain the differences seen in Bo, Cr, S, and Ti, 2-3 (Ti, 1-2 and 1-3 are again omitted from consideration). Furthermore the transfers of base bear no directional relation to the changes of base concentration in the serum. If only the four largest exchanges are considered, it may be seen that when B_s increases $B'_{c_1} - B_{c_1}$ is negative in Bo and S, but positive in Cr; when B_s drops, $B'_{c_1} - B_{c_1}$ is negative in Ti, 2-3. The analyses of cells for base, then, indicate that base is exchanged in an extremely irregular way between cells and serum.

DISCUSSION

Analysis of the experimental data presents only a paradox. From the changes of cell volume and the redistribution of water the cells seem to act as simple osmometers with membranes impermeable to base; direct chemical analyses, on the other hand, indicate that base traverses the cell membranes in a highly capricious manner which cannot usefully serve the interests of osmotic equilibrium. The first impulse, when this paradox presented itself, was to impugn the accuracy of the analytical measurements.

The estimations of serum base and serum water cannot be seriously questioned. The non-protein solids of serum are so small and constant that serum water can be estimated by the formula of Eisenman, Mackenzie, and Peters (4) with a mean error of only 0.3 per cent. As water of serum is used only as a factor to correct base concentrations, this is negligible. The error in analysis for base is usually less than 1, rarely as much as 2 milliequivalents, 0.75 and 1.5 per cent respectively. The total error in the ratio B_{s_1}/B_{s_2}, therefore, should be less than 1.5 and could not exceed 3.0 per cent. The water of cells can be estimated from oxygen capacity and cell volume by the formula of Eisenman, Mackenzie, and Peters (4) with a mean error of only 0.9 per cent. This would mean that W_{c_1}/W_{c_1} should not be at fault by more than 2 per cent. Unless these estimates of error are sadly erroneous, the agreement between the ratios of water in cells and base in serum is too satisfactory to be fortuitous.

There is no reason to believe that the error of estimating base in cells is any larger. Calculation is simple, involving only the values for cell volume and for base of whole blood and serum. If cell volume is in the neighborhood of 40 to 50 per cent, the total error in estimation of B_{c_1} or B'_{c_1} should not exceed 4 milli-equivalents. It is true that in two cases (Bo and S) with large discrepancies between B'_{c_1} and B_{c_1}, cell volumes are smaller. Even if the error increased in proportion as the cell volume diminished, the error should not reach 12 milli-equivalents.

To test this point further five experiments were conducted *in vitro*. In these sodium chloride, potassium chloride, or sodium sulfate, as dry salt, was added to one sample of blood, while an untreated sample of the same blood was used as a control. Both samples were subjected to the same analyses as the bloods from the *in vivo* experiments described above. The results, presented in Table III,[1] have been calculated by the same methods which were applied to the *in vivo* data. The changes of base and cell volume produced in the test-tube are far greater than those that were produced by intravenous injection. In four of the five experiments W_{c_2}/W_{c_1} and B_{s_1}/B_{s_2} agree within 5 per cent; in one they differ by 7 per cent. In no instance does $B'_{c_1} - B_{c_1}$ exceed 4 per cent and in only one is it greater than 2 per cent.

This establishes beyond reasonable doubt the accuracy of the analytical methods and the calculations involved in the determination of cell base. If this constitutes a valid control, transfers encountered after intravenous experiments must be accepted as real. The differences between cell water and serum base ratios are somewhat larger in the *in vitro* than in the *in vivo* experiments. This is to be expected, since errors in calculation will vary to a certain extent with the magnitude of cell volume changes. The discrepancies found are probably not outside these errors and cannot be used as arguments against the purely osmotic character of the changes of cell volume.

If these conclusions are correct, it would appear that when the concentration of salt in the circulating blood is altered, although moderate amounts of base may pass in either direction across the envelopes of the red blood cells, the latter, nevertheless, react like

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[1] Analytical data and further details of these experiments have been published elsewhere ((4) Table IV).

perfect osmometers with membranes permeable to water, but not to base. The possibility that this apparent contradiction is referable to errors of analysis or calculation cannot, of course, be definitively excluded. No such errors, however, can be discovered. It is, therefore, justifiable to suggest a tentative explanation for the paradox. The *in vitro* experiments, in which no base was transferred, are distinguished from the infusion experiments, in which base was transferred, by the fact that in the former the metabolic activities of the blood were held in abeyance. Halpern (5) has recently shown that, although neither base nor phosphate enters or leaves the red blood cell in behalf of osmotic equilibrium, phosphate can be made to pass freely between cells and serum in response to certain cellular metabolic activities in which it is con-

TABLE III

Exchanges of Water and Base between Cells and Serum of Blood after Additions of Salt in Vitro

Experi- ment No.	$\dfrac{W_{c_2}}{W_{c_1}}$	$\dfrac{B_{s_1}}{B_{s_2}}$	B_{c_1}	B'_{c_2}	$B'_{c_2} - B_{c_1}$	Added to blood	
			m.-eq.	*m.-eq.*	*m.-eq.*	*m.-eq.*	
1	0.772	0.771	100.8	101.3	0.5	NaCl	37.3
2	0.816	0.772	101.3	105.3	4.0	"	36.6
3	0.806	0.751	117.8	116.7	−1.1	KCl	37.1
4	0.806	0.767	112.3	110.4	−1.9	"	36.6
5	0.846	0.774	112.8	113.7	0.9	Na_2SO_4	38.0

cerned, carrying with it a certain quantity of potassium. It is conceivable, since the activity of potassium combined with organic phosphate is quite unknown, that exchanges of phosphate between cells and serum in connection with metabolic activities may cause little osmotic disturbance. The intravenous infusions in these experiments may well have altered metabolism profoundly, since they were invariably given for therapeutic purposes to patients who were seriously ill. In Halpern's experiments osmotic effects of transfers of phosphate were not carefully investigated. In a few preliminary studies of movements of phosphate during carbohydrate metabolism Dann and Hald (6) have found that large transfers of phosphate between cells and serum in the circulating blood cannot be correlated with changes of cell volume.

On the whole the red cells in the circulating blood seem to react as they do in the test-tube, expanding and contracting in response to osmotic influences by exchanges of water without base. At the same time, whatever its osmotic effect may be, it must be possible for base to traverse the cell membrane under particular conditions. These conditions, as far as they have been reproduced *in vitro*, appear to be connected with metabolic activity and to serve no osmotic purpose. Neglect of the distinction between resting and active blood and between osmotic and metabolic demands may be responsible for much of the controversy over the permeability of the red blood cell membrane. If this distinction is valid, the concentration of base in living cells in their natural environment must be continually changing, within limits, without direct relation to osmotic pressure. This is precisely what was suggested in a comparison of the base in cells and serum of normal individuals (2). The concentrations of potassium, sodium, and base in cells were quite variable and not consistently related to the concentrations of the same substances in serum.

In the experiments thus far presented estimations of water transferred have been somewhat short of exact because the quantities of water originally in the cells were not measured directly, but calculated from concentrations of hemoglobin by an empirical formula. Consequently it is impossible to state unequivocally that discrepancies between the ratios W_{c_2}/W_{c_1} and B_{s_1}/B_{s_2} were referable entirely to analytical error and that the larger differences, at least, had no significance. In a series of *in vitro* experiments, some of which have been reported earlier, in which water or salts of various kinds were added to blood, the water of serum and blood was measured directly by drying the materials to constant weight. The results of these experiments are shown in Table IV. In a few instances, especially in the a series, the discrepancies between the two ratios are unexpectedly large, without any apparent relation to the treatment which the blood received. In no experiment could any transfer of base be detected by analyses of serum. Whether the large discrepancies have any significance and denote changes in the activities of cell base it is impossible to say, although it seems highly improbable. That the average ratio $(W_{c_2}/W_{c_1}):(B_{s_1}/B_{s_2})$ equals 1.00 is far more important. It has been claimed that as much as 30 per cent

of the water of cells is "bound"; *i.e.*, is not available as solvent (*e.g.* (7)).   Macleod and Ponder (8) have already called attention to the fact that estimations of "bound" water were unfortunately planned, since the substances used for its determination, glycerol, etc., were not inert solutes.   From the results of the experiments in Table IV all the water of cells and serum appears to be available as solvent for the salts which were added and, judging from the effects of adding water, for the solutes already present in blood.

TABLE IV

Relations of Transfers of Water between Cells and Serum to Changes of Serum Base after Addition to Blood in Vitro of Salts or Water

Experiment No.[*]	$\dfrac{W_{c_2}}{W_{c_1}}$ (1)	$\dfrac{B_{s_1}}{B_{s_2}}$ (2)	(1)/(2)	Added to blood
1	0.800	0.798	1.00	34.7 m.-eq. NaCl and KCl
4	0.849	0.825	1.03	31.3 " "
5	0.817	0.821	1.00	32.2 " KCl
2	0.928	0 942	0.99	9 2 " Na_2SO_4 and K_2CO_3
3	0.869	0.938	0.93	13.9 " " " "
6	0.906	0 907	1.00	12.9 " Na_2CO_3
7	1.334	1.293	1.03	250 cc. H_2O
8	1.260	1.305	0.97	250 " "
1-*a*	0.934	0.926	1.01	11.5 m.-eq. K_2CO_3
2-*a*	0.865	0.757	1.14	35.6 " KCl
3-*a*	0.816	0.779	1.05	35.7 " NaCl
4-*a*	1 189	1.268	0.94	200 cc. H_2O
5-*a*	1.230	1.322	0.93	250 " "
6-*a*	0.871	0.882	0.99	19.7 m.-eq. Na_2CO_3
Average.................		1.001		

* Experiments 1 to 8 are from Wakeman, Eisenman, and Peters (1); the remainder have not been published previously.

SUMMARY

The exchanges of water and base between cells and serum in the circulating blood of patients after intravenous injections of hypertonic solutions of sodium salts have been examined.

The red blood cells seem to act as simple osmometers with membranes impermeable to base, swelling and contracting in proportion to changes of the concentration of base in the water of serum.   Nevertheless significant quantities of base seem to cross

the cell membranes.   The apparent paradox cannot be attributed to discoverable analytical errors.   It is suggested that base may be transferred in behalf of cellular metabolic activities rather than osmotic adjustments.

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ON THE STRUCTURE OF PROTEINS: CATTLE HEMO-
GLOBIN, EGG ALBUMIN, CATTLE FIBRIN,
AND GELATIN

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The existence of simple numerical relationships among the main amino acids of gelatin and cattle fibrin has been pointed out in previous papers (1, 2). In this communication the study has been extended to include two other proteins, *i.e.* cattle hemoglobin and egg albumin.

For the analysis of hemoglobin we have, as in earlier examples, restricted ourselves to the determination of those amino acids for which reliable analytical methods were available. The percentage composition thus obtained is presented in Column 1 of Table I. These values were recalculated on a gm. molecular basis and are given in Column 3. The ratios in Column 5 were obtained directly from Column 3. On examination of Column 5 it is seen that cattle globin on acid hydrolysis produces equivalent amounts of arginine, proline, tyrosine, and of histidine and aspartic acid. These facts suggest the existence of a basic law which must have a rôle in the interpretation of the structure of the globin molecule.

The average molecular weight of the amino acids that are liberated on the complete hydrolysis of globin (4) was estimated to be 135.6, and from this it follows that the average residue weight is 117.6.[1] From this or the corrected value (115.5) it can readily be calculated that 100 gm. of globin must give approximately 0.865

[1] This value is undoubtedly too high because the amino acids which can be estimated with relative accuracy are those the molecular weights of which are in the higher brackets. Therefore, more weight should be given to those amino acids which cannot be determined with exactitude. By such a procedure the average residue weight of the units in globin was found to be 115.5.

(0.851 uncorrected) gm. molecule of an average amino acid on complete hydrolysis. On considering the values in Column 3 one finds that the various amino acids listed in Table I comprise 1/16, 1/18, 1/18, 1/36, 1/48, 1/48, 1/48, and 1/192 of all of the constituent amino acids.

Examination of the ratios in Column 5 and the fractional values in Column 6 leads to the conclusion that cattle globin must contain 576 amino acid residues or a whole number multiple thereof. When the number of units is multiplied by the average residue

TABLE I

Ratio of Amino Acids in Hemoglobin after Hydrolysis

Amino acid	Weight	Mol. wt.	Gm. molecule per 100 gm. protein		Ratio	Fraction of total residues (frequency)
			Found	Calculated*		
	(1)	(2)	(3)	(4)	(5)	(6)
	per cent					
Lysine..................	8.0	146	0.054_6	0.054_6	36	16
Histidine......	7 4	155	0.047_8	0.048_5	32	18
Aspartic acid	6.4	133	$0 047_9$	$0 048_5$	32	18
Glutamic "	3.5	147	0.023_9	0.024_2	16	36
Tyrosine.	3.3†	181	0.018_2	0.018_2	12	48
Proline..................	2.1	115	0.018_2	$0 018_2$	12	48
Arginine	3.1	174	0.018_1	$0 018_2$	12	48
Cysteine‡	0.5_6	121	0.004_6	0.004_6	3	192

* Base = 0.018_2 (gm. molecule of tyrosine, arginine, and proline).

† See Roche (3).

‡ In this and Tables II and III the cystine-cysteine sulfur has been calculated as cysteine.

weight (115.5), it is found that cattle globin has a minimum molecular weight of 66,500. As hemoglobin contains 4.2 per cent of heme, its molecular weight is therefore about 69,000. Northrop and Anson (5) determined the molecular weight of cattle carbon monoxide hemoglobin by the diffusion method and obtained the value 68,600 ± 1000.[2]

It should be pointed out that the molecular weight given above was obtained without the aid of any suppositions. The single

[2] A molecular weight of the same magnitude has been obtained by other investigators employing different methods; see Roche (3).

uncertainty is the estimation of the average residue weight, as this involves a consideration of amino acids which at present cannot be isolated in a quantitative manner. As for the other values used in the calculation, it is evident upon examination of Columns 3 and 4 that the agreement between the theoretical and found values is within 2 per cent.

Recently, crystalline egg albumin has been analyzed by Calvery (6), Vickery and Shore (7), and Baernstein (8), and their results have been employed in the compilation of Table II. It is evident that eight of the amino acids of egg albumin are present in amounts described by the ratio $36:16:12:12:12:8:4:4$. As the molecular

TABLE II

Ratio of Amino Acids in Egg Albumin after Hydrolysis

Amino acid	Author	Weight	Mol. wt.	Gm. molecule per 100 gm. protein		Ratio	Fraction of total residues (frequency)
				Found	Calculated*		
	(1)		(2)	(3)	(4)	(5)	(6)
		per cent					
Glutamic acid.......	6	14.0	147	0.095_2	0.101_4	36	8
Aspartic "	6	6.1	133	0.045_8	0.045_1	16	18
Methionine..........	8	5.2	149	0.034_9	0.033_8	12	24
Lysine..............	7	5.0	146	0.034_2	0.033_8	12	24
Arginine......... ...	7	5.6	174	0.032_2	0.033_8	12	24
Tyrosine............	6	4.2	181	0.023_2	0.022_5	8	36
Histidine............	7	1.5	155	0.009_7	0.011_2	4	72
Cysteine............	6	1.3	121	$0\ 010_8$	0.011_2	4	72

* Base = 0.033_8 (average gm. molecule of methionine, lysine, and arginine).

weight of the average amino acid was estimated to be 142 (4, 6–8) and the average residue weight to be 124, it follows that 100 gm. of egg albumin should yield on hydrolysis 0.806 gm. molecule of the hypothetical average amino acid. With this value, the numerical fractions, given in Column 6, were obtained. Granting the validity of these experimental results, it is obvious that egg albumin contains 288 amino acid residues or a whole number multiple thereof. If this value is multiplied by the average residue weight, it follows that egg albumin has a minimum molecular weight of 35,700, which is in good agreement with Svedberg's value of 34,500, determined with the aid of the ultracentrifuge (9).

The same method of calculation has been applied to our previously reported analysis of cattle fibrin (1). Approximately 84 per cent of hydrolysis products have been obtained from fibrin (1, 4), and, as these units have an average residue weight of 120.3, it follows that glutamic acid, lysine, arginine, aspartic acid, proline, tryptophane, histidine, methionine, and cysteine comprise 1/8, 1/12, 1/18, 1/18, 1/18, 1/32, 1/48, 1/48, and 1/64 of all of the amino acid residues. On consideration of these values and the previously reported ratios it is evident that the cattle fibrin molecule contains 576 units or a whole number multiple thereof. From this value and that of the average residue weight the molecular weight of cattle fibrin was found to be approximately 69,300 or a whole number multiple thereof.

In the case of gelatin 103 per cent of the amino acids are known (1, 10) and the average residue weight is approximately 90. As gelatin has been shown to be heterogeneous (11), it is not proper to consider a calculation of chain length or molecular weight. However, it appears likely that the precursor of gelatin contains a chain of 288 units or a whole number multiple thereof. This value would lead to a minimum molecular weight of approximately 26,000. Atkin (12) has calculated, on the basis of Dakin's analysis (10) and with the assumption that the molecule contains 2 moles of histidine (0.9 per cent), that gelatin has a molecular weight of 36,400 and contains 360 amino acid residues. Adair (13) calculated a molecular weight of 68,000 ± 2000 from the osmotic pressure measurements of Lillie (14), but Krishnamurti and Svedberg (11), employing the ultracentrifuge, observed the existence of particles varying from 10,000 to 70,000 (see also Lansing and Kraemer (15)). It is apparent that a definite opinion must be deferred until homogeneous preparations of gelatin are available for analysis.

In the case of cattle hemoglobin and egg albumin the chemical method and the various physical methods give molecular weights which are in good agreement. As the physical methods tend to give maximum values and the chemical method minimum values, it appears likely that a true molecular weight is indicated when these two procedures yield results of the same order of magnitude.

In 1934 Block (16) found a constant ratio of iron to arginine to histidine to lysine of 1:3:8:9 in horse, sheep, and dog hemoglobins

and in a subsequent paper (17) described partial formulæ which contained 13 moles of arginine, 33 moles of histidine, 37 moles of lysine, and 4 moles of iron per mole of protein (see also (6, 7, 18–20)). The experimental values of Block (16) and those of Vickery and White (21) have been recalculated and are presented in Table III.

Examination of Table III reveals that the various hemoglobins conform to similar numerical rules and therefore in general exhibit similar structural patterns. As the structures are not absolutely identical, the hemoglobins of different species appear to differ from one another in several respects, *i.e.* the sulfur-containing constituents.

TABLE III

Ratio of Constituents in Various Hemoglobins

Constituent	Hemoglobin			
	Horse	Sheep	Cattle	Dog
Iron.........	4	4	4	4
Cysteine....	2	3	3	6
Non-cysteine sulfur...	6	12		6
Arginine......	12	12	12	12
Histidine.......	32	32	32	32
Lysine................	36	36	36	36

It is well known that the experiments of Svedberg (22–24) on the particle size of proteins have given in many cases values that are approximately whole number multiples of 34,500. Svedberg has advanced no physical or chemical explanations for the dominant rôle of this numerical value. Astbury and Woods (25) have suggested that the maximum length of a peptide chain must be limited by the vibrational instability of the structure, the fundamental magnitude of 34,500 being the ultimate possible chain length consistent with vibrational stability.[3] Rimington (26), on the other hand, offers the suggestion that the elementary complex of protein systems, in general, consists of a number of peptide chains grouped together and held by covalent forces in micelles corresponding to a molecular weight of 34,500.

[3] If this hypothesis is correct, one would expect the molecular weight to vary with the temperature, all other factors remaining constant.

Our researches indicate that the "Svedberg unit" is a consequence of a law governing the structure of protein molecules. The following facts are offered for consideration. Cattle globin, as it exists in hemoglobin, contains 576 or $2^6 \times 3^2$ amino acid residues; egg albumin, 288 or $2^5 \times 3^2$ units; and cattle fibrin, 576 or $2^6 \times 3^2$ units. The number of accurately determined amino acid residues per molecule of cattle globin, cattle fibrin, or egg albumin (Column 5 of Tables I and II) can be arranged as in Table IV. The fractional amount of the individual amino acids in respect to the total number present in the molecule (Column 6 of Tables I and II) obviously can be presented in a similar manner (Table V).[4]

TABLE IV

Number of Individual Amino Acid Residues per Molecule of Protein

Group	$2^0 \times 3^m$	$2^1 \times 3^m$	$2^2 \times 3^m$	$2^3 \times 3^m$	$2^4 \times 3^m$	$2^5 \times 3^m$
$2^n \times 3^0$			2^2	2^3	2^4	2^5
$2^n \times 3^1$	3		$2^2 \times 3$		$2^4 \times 3$	
$2^n \times 3^2$	3^2	2×3^2	$2^2 \times 3^2$	$2^3 \times 3^2$		

TABLE V

Fractional Amount of Individual Amino Acid Residues in Relation to Total Number of Residues per Molecule of Protein

Group	$2^1 \times 3^m$	$2^2 \times 3^m$	$2^3 \times 3^m$	$2^4 \times 3^m$	$2^5 \times 3^m$	$2^6 \times 3^m$
$2^n \times 3^0$			2^3	2^4	2^5	2^6
$2^n \times 3^1$		$2^2 \times 3$	$2^3 \times 3$	$2^4 \times 3$		$2^6 \times 3$
$2^n \times 3^2$	2×3^2	$2^2 \times 3^2$	$2^3 \times 3^2$			

The structure of fibrin, globin, egg albumin, and the precursor of gelatin is such that the total number of amino acid residues in the protein molecule is $2^n \times 3^m$, where n and m are whole numbers, and the number of individual amino acid residues is $2^{n_1} \times 3^{m_1}$, where n_1 and m_1 may be either 0 or a whole number. The significance of the Svedberg unit lies in the fact that many, if not all, genuine protein molecules contain $n \times 288$ units, where n is a whole number other than 0. With an average residue weight of

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[4] The amino acids of gelatin that have been determined with the more reliable methods also fit into this arrangement.

120, a molecular weight of approximately 34,500 or a multiple thereof results. From this it follows that the Svedberg unit is not an absolute value but is dependent upon the average residue weight of the constituent amino acids. With proteins such as gelatin and silk fibroin which contain large quantities of amino acids with low residue weights one would expect considerable deviation from the Svedberg unit.

In general, it appears that those proteins, including such substances as insulin (27), thyroglobulin (28), Bence-Jones protein (29), pepsin (30), trypsin (31), and antibodies (32), the particle size of which is equivalent to the Svedberg unit or a whole number multiple thereof (22–24), contain $2^n \times 3^m$ amino acid residues.

In an attempt to explain the numerical rules which appear to govern the structure of the protein molecule, the hypothesis has been proposed (1, 2) that in every protein each amino acid residue is distributed throughout the entire peptide chain at constant intervals; *i.e.*, each amino acid residue recurs with a characteristic whole number frequency.[5] For example, every one of the thirty-six lysine residues in cattle globin is separated from the previous and succeeding one by fifteen other residues, and similarly every one of the twelve proline residues is separated by forty-seven other residues. The protein molecule therefore contains a great number of superimposed frequencies. When it is realized that these frequencies refer to position numbers, it is evident that the protein molecule cannot be disintegrated into fragments of identical structure. Although similar combinations of a few amino acid residues may occur at certain intervals along the peptide chain, in every case each combination will lie in a different environment. Therefore, no 2 units in the peptide chain possess identical structural significance. From these observations it is evident that only a limited number of homogeneous peptides can be obtained from a given protein, and that the fractional amount of a particular amino acid that can be obtained from a protein in the form of a homogeneous peptide will decrease as the chain length of the desired peptide increases.

In an earlier section it was pointed out that the number of

[5] The term "periodicity" employed in earlier papers (1, 2) has been changed to the more descriptive "frequency."

units in a molecule of globin, fibrin, or egg albumin was $n \times 288$. While this value in itself is important, when considered in conjunction with the frequency hypothesis, it acquires additional significance, as illustrated by the following example. When the residues contained in cattle globin are arranged according to their respective frequencies, it becomes apparent that the structural pattern will vary until 576 units are present. The pattern then starts to repeat itself and if 1152 units are considered it is noticed that the second 576 units can be superimposed upon the first 576. Thus the term $n \times 288$ defines the number of amino acid residues necessary to complete the unique structural pattern of the protein in question.

The universal association of proteins with living processes has led to the conclusion that the phenomenon of life is dependent upon the presence of proteins. This idea gains support through the knowledge that proteins exhibit a degree of organization found in no other substance of the inorganic or organic realms. Although this organization is precise, at the same time it offers sufficient latitude for biological variations which are exhibited, for example, by the hemoglobins. The existence of this highly developed organization within the protein molecule suggests the presence or need of chemical organizers for the synthesis of individual proteins. The histological differentiation of living organisms has led to the conception of organizers, and this conception can be transferred to the chemical domain when it is assumed that an organizer is required in the formation of proteins. The ability of individuals to synthesize clearly defined proteins in normal and pathological metabolism and the transmission of this ability through inheritance and infection[6] cannot be explained in any other way than through the assumption that organizers are required for the synthesis of proteins. In conjunction with our experiments on enzymatic specificity, an attempt is being made to determine whether or not enzymes can function as organizers in the synthesis of individual proteins.

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[6] Stanley (33) has pointed out that tobacco plants after infection with the tobacco mosaic virus form large amounts of a crystalline protein and that this protein can in itself produce further infection. The question arises as to whether the protein itself acts as an organizer or whether during the infection an organizer is introduced with the protein.

Preparation of Hemoglobin Hydrolysate—800 gm. of cattle hemoglobin (Eastman, Practical grade) were refluxed with 4 liters of 20 per cent hydrochloric acid for 24 hours. After cooling, the acid-insoluble residue was removed and washed with cold water, and the combined filtrate and washings were concentrated to a thick syrup. This residue was repeatedly taken up in water and evaporated until the major portion of the hydrochloric acid had been volatilized. The hydrolysate was then made up to 2 liters. 100 cc. of this solution were equivalent to 35.45 gm. of moisture- and ash-free protein containing 17.0 per cent nitrogen, *e.g.* globin (34).

d-Arginine—200 cc. of hydrolysate (70.9 gm. of protein) were diluted to 600 cc. and the solution was adjusted to pH 3 by the cautious addition of silver oxide. After the precipitated silver chloride was removed and washed, the solution (1000 cc.) was heated to 90° and treated with 100 cc. of water containing 10.0 gm. of flavianic acid. After standing in the cold room for several days the flavianate was collected and recrystallized first from 1200 cc. and then from 1400 cc. of hot water containing a trace of flavianic acid. The twice recrystallized flavianate was then recovered, washed with a small quantity of absolute ethanol, and dried to constant weight at 105°. Yield, 5.93 gm.

$(C_{10}H_6N_2SO_8) \cdot (C_6H_{14}O_2N_4)$.   Calculated, N 17.2; found, N 17.1

When correction is made for the solubility of arginine flavianate in 2.6 liters of solvent (35), the yield is raised to 6.37 gm., which is equivalent to 2.27 gm. of arginine or 3.1 per cent of the protein.

d-Lysine—200 cc. of hydrolysate were diluted to 600 cc., the solution was adjusted to pH 3 with silver oxide, and the silver chloride was removed and washed in the usual manner. An excess of silver nitrate was added to the filtrate which was then adjusted to pH 13 by the addition of warm, saturated barium hydroxide. After the precipitate was removed and washed with dilute baryta, the filtrate was freed of barium, with sulfuric acid, and saturated with hydrogen sulfide. The precipitate was removed and washed, and the filtrate was concentrated to 1 liter. 50 gm. of sulfuric acid were then added and the lysine was precipitated from the hot

solution (90°) by the addition of an excess of phosphotungstic acid dissolved in 5 per cent sulfuric acid. After standing in the cold room for 3 days, the lysine phosphotungstate was collected and washed with a cold solution of 2 per cent phosphotungstic acid in 5 per cent sulfuric acid. The volume of the washings and filtrate was 2100 cc. The precipitate was dissolved in aqueous acetone (1:1) and decomposed with barium hydroxide. After the barium phosphotungstate was removed and washed with dilute baryta, the filtrate was adjusted to pH 3 with dilute sulfuric acid. The barium sulfate was removed and the filtrate concentrated to 250 cc. The major portion of the residual sulfuric acid was removed by the cautious addition of barium hydroxide and the filtrate was concentrated to 50 cc. in the presence of a slight excess of barium carbonate. After the precipitate was removed and washed, the filtrate was reduced to 25 cc., and sufficient absolute ethanol was added to produce a permanent turbidity. 10.0 gm. of picric acid in 50 cc. of warm ethanol were then added to the lysine solution and the reaction mixture was placed in the cold room overnight. The precipitated lysine picrate was collected, washed, dried, and weighed in the usual manner. Yield, 13.80 gm. of lysine picrate, m.p. 265° with decomposition, or 5.38 gm. of lysine.

$(C_6H_3O_7N_3)\cdot(C_6H_{14}O_2N_2)$.   Calculated, N 18.7; found, N 18.7

When corrected for the solubility of lysine phosphotungstate in 2100 cc. of solution (1), the yield is raised to 5.67 gm. or 8.0 per cent of the protein. A second estimation gave 8.0 per cent.

l-Histidine—150 cc. of hydrolysate (53.17 gm. of protein) were diluted to 600 cc., the solution was adjusted to pH 3 by the addition of silver oxide, and the silver chloride was removed and washed in the usual manner. An excess of silver nitrate was added to the filtrate which was then adjusted to pH 7.6 with warm, saturated barium hydroxide. The precipitate was recovered, suspended in water acidulated with sulfuric acid, and decomposed with hydrogen sulfide. After the silver sulfide-barium sulfate precipitate was removed, the solution was concentrated to 300 cc., and the excess sulfuric acid was removed by the cautious addition of dilute baryta. The solution was freed of barium sulfate and made up to 500 cc. 25 gm. of sulfuric acid and an excess of 10 per cent of mercuric sulfate in 5 per cent sulfuric acid were then added

and the reaction mixture was placed in the cold room for 3 days. The histidine-mercury precipitate was recovered, suspended in water, and decomposed with hydrogen sulfide. After the mercuric sulfide was removed and washed, the solution was concentrated to 300 cc. and adjusted to pH 4 with cold, saturated baryta. The barium sulfate was filtered off and washed, and the filtrate was concentrated to 150 cc. 12.1 gm. of flavianic acid in 125 cc. of warm water were added to the histidine solution and, after the mixture stood at room temperature for several days, the flavianate was collected and dried on clay. Yield, 12.50 gm. On drying at 105° the air-dry substance lost 10.1 per cent moisture and the anhydrous substance, m.p. 224–226° with decomposition, possessed the following composition.

$(C_{10}H_6N_2SO_8)\cdot(C_6H_9N_3O_2)$. Calculated. C 40.9, H 3.2, N 14.9
                                             Found.   " 40.9, " 3.0, " 15.1

When converted to an anhydrous basis, allowance being made for the solubility of histidine monoflavianate (35), the yield is raised to 11.80 gm. of histidine monoflavianate or 3.90 gm. of histidine or 7.3 per cent of the protein. A second estimation gave 7.4 per cent.

l-Aspartic Acid—200 cc. of hydrolysate were diluted to 1 liter and the basic amino acids were removed with phosphotungstic acid, as directed by Van Slyke (36). The filtrate from the phosphotungstic acid precipitation was freed of excess reagent by extraction with amyl alcohol and ether, and the resulting solution of monoamino acids was evaporated to a thick syrup. The syrupy residue was taken up in 300 cc. of water and the glutamic and aspartic acids were separated as their barium salts (37). On decomposition of the crude barium salts and after the solution was freed of inorganic ions, the aspartic acid was isolated as the copper salt with the aid of cupric acetate. The copper aspartate was collected on a filter, washed with ethanol, and dried at 105° for 6 hours. Yield, 7.93 gm.

$C_4H_5O_4NCu\cdot2H_2O$. Calculated, N 6.0; found, N 6.0

7.93 gm. of cupric aspartate dihydrate are equivalent to 4.53 gm. of aspartic acid or 6.4 per cent of the protein. A second estimation gave 6.4 per cent.

d-Glutamic Acid—The filtrate from the aspartic acid separation was saturated with hydrogen sulfide, the copper sulfide removed, and the filtrate concentrated to 25 cc. The concentrate was then saturated with hydrogen chloride and placed in the cold room overnight. The glutamic acid hydrochloride that had separated was collected and dried to constant weight over potassium hydroxide. Yield, 2.82 gm.

$C_5H_{10}O_4NCl$. Calculated, NH_2-N 7.6; found, NH_2-N 7.5

The mother liquor resulting from the first crystallization of the glutamic acid hydrochloride was heated on the steam bath for 8 hours, cooled to 0°, and saturated with hydrogen chloride. On standing in the cold room for several days, an additional 0.30 gm. of glutamic acid hydrochloride was recovered. Thus the total yield of glutamic acid hydrochloride was 3.12 gm. or 2.50 gm. of glutamic acid or 3.5 per cent of the protein.

l-Proline—800 cc. of hydrolysate (283.6 gm. of protein) were concentrated to a thick syrup. When the residue was taken up in a small quantity of warm water and 2 volumes of ethanol were added, a precipitate formed which was removed and dried on clay at room temperature. Yield, 107.6 gm.[7] The filtrate was again concentrated to a syrup and taken up in 1500 cc. of 90 per cent ethanol. After standing for several days in the cold room the precipitate that had formed was collected and dried as above. Yield, 69.3 gm. The filtrate was concentrated and taken up in 600 cc. of 95 per cent ethanol and placed in the cold room for 3 days. The precipitate was recovered, yield 37.8 gm., and the filtrate concentrated to a thick syrup. This residue was then made up to 500 cc. with 0.5 N hydrochloric acid. 250 cc. of this prepared hydrolysate and 21.0 gm. of ammonium rhodanilate in 150 cc. of methanol were shaken on the machine for 24 hours. After standing in the cold room for 6 hours, the homogeneous crystalline precipitate was collected and dried in the usual manner (1). Yield, 15.0 gm.

$(C_{16}H_{14}N_6S_4Cr)\cdot(C_5H_{10}O_2N)\cdot H_2O$.   Calculated. C 41.7, H 4.3
                                                         Found.   " 41.8, " 4.4

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[7] Examination of this fraction revealed the presence of tyrosine and leucine. The former was isolated as the free amino acid (N, 7.7) and the latter as the N-carbamyl derivative (N, 16.2).

When corrected for the minimum solubility of proline rhodanilate in the mother liquor, the yield is raised to 15.75 gm:, which is equivalent to 3.00 gm. of proline or 2.1 per cent of the protein.

l-Cystine—17.7 gm. (corrected) of cattle hemoglobin were hydrolyzed with 200 gm. of 8 N sulfuric acid in the presence of tin for 24 hours. The hydrolysate was cooled and filtered, and the filtrate was made up to 1 liter. 300 cc. of hydrolysate were withdrawn and the cysteine was determined by the cuprous mercaptide method of Vickery and White (38). In the first experiment 56.9 mg. of barium sulfate were obtained, which is equivalent to 29.3 mg. of cystine or 0.55 per cent of the protein. In a second experiment, with 290 cc. of hydrolysate, 54.4 mg. of barium sulfate were isolated, which is equivalent to 28.0 gm. of cystine or 0.54 per cent of the protein.

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CONFIGURATIONAL RELATIONSHIP OF MANDELIC ACID TO LACTIC ACID

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The configurations of the aliphatic α-hydroxy acids have not been previously correlated to those of the acids containing a phenyl group by the classical methods of organic chemistry. Recently, however, Levene and Harris[1] correlated secondary aliphatic alcohols with the secondary alcohols containing a phenyl group.

The configurations of the aliphatic secondary alcohols were correlated to those of the aliphatic α-hydroxy acids by the use as an intermediate of the unsaturated alcohol which, on one hand, was hydrogenated to the saturated carbinol, and on the other, was oxidized to an α-hydroxy acid. This method now has been applied to the correlation of propylcyclohexylcarbinol to hexahydromandelic acid. For convenience of operation, the acetyl derivative of the unsaturated carbinol was used for oxidation to acetylhexahydromandelic acid. These reactions are expressed in Formulæ V to VIII. The relationships of mandelic acid to acetylhexahydromandelic acid are given in Formulæ IX to XI. Formulæ V to XI thus establish the relationship between levo-propylcyclohexylcarbinol and levo-mandelic acid. The relationship between levo-propylcyclohexylcarbinol and levo-propylphenylcarbinol is given by Formulæ I and V. Thus the conclusion is reached that levo-propylphenylcarbinol is correlated to levo-mandelic acid.

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[1] Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **113**, 55 (1936).

$$
\begin{array}{ccccccc}
& \text{COOH} & & \text{C}_3\text{H}_7 & & \text{C}_3\text{H}_7 & & \text{COOH} \\
& | & & | & & | & & | \\
\text{H}\cdots & \text{C} & \cdots\text{OH} \rightarrow \text{H}\cdots & \text{C} & \cdots\text{OH} \leftrightarrow \text{H}\cdots & \text{C} & \cdots\text{OH} \rightarrow \text{H}\cdots & \text{C} & \cdots\text{OH} \\
& | & & | & & | & & | \\
& \text{C}_6\text{H}_5 & & \text{C}_6\text{H}_5 & & \text{C}_6\text{H}_{13} & & \text{C}_6\text{H}_{13} \\
& \text{Levo} & & \text{Levo} & & \text{Dextro} & & \text{Levo}
\end{array}
$$

$$
\begin{array}{ccccccc}
& \text{COOH} & & \text{CH}_3 & & \text{CH}_3 & & \text{COOH} \\
& | & & | & & | & & | \\
\text{H}\cdots & \text{C} & \cdots\text{NH}_2 \rightarrow \text{H}\cdots & \text{C} & \cdots\text{NH}_2 \rightarrow \text{H}\cdots & \text{C} & \cdots\text{NH}_2 \rightarrow \text{H}\cdots & \text{C} & \cdots\text{NH}_2 \\
& | & & | & & | & & | \\
& \text{C}_6\text{H}_5 & & \text{C}_6\text{H}_5 & & \text{C}_4\text{H}_9 & & \text{C}_4\text{H}_9 \\
& \text{Levo} & & \text{Levo} & & \text{Dextro} & & \text{Levo}
\end{array}
$$

$$
\begin{array}{cccc}
\text{C}_3\text{H}_7 & \text{C}_3\text{H}_7 & \text{COOH} & \text{COOH} \\
| & | & | & | \\
\text{H}\cdots\text{C}\cdots\text{OH} \leftrightarrow \text{H}\cdots\text{C}\cdots\text{OH} \rightarrow \text{H}\cdots\text{C}\cdots\text{OH} \rightarrow \text{H}\cdots\text{C}\cdots\text{OH} \\
| & | & | & | \\
\text{C}_6\text{H}_5 & \text{C}_6\text{H}_{13} & \text{C}_6\text{H}_{13} & \text{CH}_3 \\
\text{Levo} & \text{Dextro} & \text{Levo} & \text{Levo} \\
\text{I} & \text{II} & \text{III} & \text{IV}
\end{array}
$$

$$
\begin{array}{cccc}
 & \text{CH}_3 & \text{CH} & \\
 & | & | & \\
 & \text{CH} & \text{CH} & \\
 & \| & \| & \\
\text{C}_3\text{H}_7 & \text{CH} & \text{CH} & \text{COOH} \\
| & | & | & | \\
\text{H}\cdots\text{C}\cdots\text{OH} \leftarrow \text{H}\cdots\text{C}\cdots\text{OH} \rightarrow \text{H}\cdots\text{C}\cdots\text{OCOCH}_3 \rightarrow \text{H}\cdots\text{C}\cdots\text{OCOCH}_3 \\
| & | & | & | \\
\text{C}_6\text{H}_{11} & \text{C}_6\text{H}_{11} & \text{C}_6\text{H}_{11} & \text{C}_6\text{H}_{11} \\
-16.1 & -16.0 & +12.8 & +22.2 \\
\text{V} & \text{VI} & \text{VII} & \text{VIII}
\end{array}
$$

$$
\begin{array}{ccc}
\text{COOH} & \text{COOH} & \text{COOH} \\
| & | & | \\
\text{H}\cdots\text{C}\cdots\text{OH} \rightarrow \text{H}\cdots\text{C}\cdots\text{OCOCH}_3 \rightarrow \text{H}\cdots\text{C}\cdots\text{OCOCH}_3 \\
| & | & | \\
\text{C}_6\text{H}_5 & \text{C}_6\text{H}_5 & \text{C}_6\text{H}_{11} \\
-210 & -283 & +35.4 \\
\text{IX} & \text{X} & \text{XI}
\end{array}
$$

Furthermore, from Formulæ I to IV it can be seen that levo-propylphenylcarbinol is correlated to dextro-propylhexylcarbinol and to levo-α-hydroxyoctanoic acid and hence to levo-lactic acid. Thus it follows that:

First, the configurationally related phenylated secondary alcohols and the phenylated α-hydroxy acids rotate in the same direction.

Second, configurationally related mandelic acid and α-hydroxyoctanoic acid rotate in the same direction.

Third, in the case of levo-mandelic acid as in that of the levo-α-hydroxyaliphatic acids, the shift of the direction of rotation is to the right on passing from the undissociated acid to its ionized state.

In conclusion, it may be stated that the relationship between the hydroxy derivatives discussed in this communication is similar to the relationship of the corresponding amino derivatives, as was recently demonstrated by Levene and Mardashew.[2]

EXPERIMENTAL

Levo-1-Cyclohexyl-2-Butene-1-Ol—The inactive 1-cyclohexyl-2-butene-1-ol was prepared from cyclohexyl magnesium bromide and crotonaldehyde. The carbinol was resolved by recrystallizing the brucine salt of the acid phthalic ester from 80 per cent aqueous acetone. After five recrystallizations a carbinol was obtained, from the more insoluble salt, which had a boiling point of 108–109°, $p = 15$ mm. $d_4^{25} = 0.9064$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-9.40°}{1 \times 0.906} = -10.4°; \; [M]_D^{25} = -16.0° \text{ (homogeneous)}$$

3.895 mg. substance: 11.120 mg. CO_2 and 4.100 mg. H_2O
$\qquad$ $C_{10}H_{18}O$.   Calculated.   C 77.85, H 11.77
$\qquad$ 154.1   Found.   " 77.85, " 11.77

Levo-1-Cyclohexyl-1-Butanol[3]—5 gm. of 1-cyclohexyl-2-butene-1-ol, $[\alpha]_D^{25} = -10.4°$ (homogeneous), were dissolved in 20 cc. of methanol and 0.3 gm. of Adams' catalyst was added. The suspension was shaken with hydrogen at a pressure of 3 atmospheres for 4 hours. The catalyst was filtered off, and the filtrate was

[2] Levene, P. A., and Mardashew, S., *J. Biol. Chem.*, **117**, 707 (1937).

[3] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 379 (1932).

distilled at atmospheric pressure to remove the methanol. The
residue distilled at 128°, $p = 38$ mm.

$$[\alpha]_D^{20} = \frac{-9.30°}{1 \times 0.90} = -10.3°; \ [M]_D^{25} = -16.1° \ (\text{homogeneous})$$

3.025 mg. substance: 8.525 mg. CO_2 and 3.500 mg. H_2O
$C_{10}H_{20}O$. Calculated. C 76.84, H 12.91
156.2   Found.   " 76.85, " 12.94

Dextro-1-Acetoxy-1-Cyclohexyl-2-Butene—40 gm. of 1-cyclohexyl-
2-butene-1-ol, $[\alpha]_D^{25} = -10.4°$ (homogeneous), were dissolved in
27 gm. of pyridine and cooled.   Then 27 gm. of acetic anhydride
were slowly added.   The solution was allowed to stand overnight
at 20°.   It was then poured into ice water and extracted with
ether.   The extract was washed with dilute sulfuric acid, water,
and dilute potassium carbonate solution, and dried over calcium
sulfate.   The acetate was distilled.   B.p. 87°, $p = 1$ mm.; yield,
47 gm. $d_4^{25} = 0.9447$ (*in vacuo*). $n_D^{25} = 1.4607$.

$$[\alpha]_D^{25} = \frac{+6.15°}{1 \times 0.945} = +6.51°; \ [M]_D^{25} = +12.8° \ (\text{homogeneous})$$

4.538 mg. substance: 12.250 mg. CO_2 and 4.110 mg. H_2O
$C_{12}H_{20}O_2$. Calculated. C 73.41, H 10.28
196.2   Found.   " 73.61, " 10.13

Dextro-2-Acetoxy-2-Cyclohexylacetic Acid—30 gm. of 1-acetoxy-1-
cyclohexyl-2-butene, $[\alpha]_D^{25} = +6.51°$ (homogeneous), were dis-
solved in 500 cc. of acetone, and powdered potassium permanga-
nate was added until the color persisted upon heating to the boiling
point.   The precipitate was filtered and washed with hot water;
the filtrate was decolorized with sodium bisulfite solution and
neutralized with sodium carbonate.   This was concentrated to a
small volume under reduced pressure, acidified with dilute sulfuric
acid, and extracted with ether.   The extract was dried over an-
hydrous sodium sulfate.   After removal of the ether, the residue
distilled at 135–140°, $p = 0.3$ mm.

$$[\alpha]_D^{25} = \frac{+1.55° \times 100}{1 \times 14.0} = +11.1°; \ [M]_D^{25} = +22.2° \ (\text{benzene})$$

3.408 mg. substance: 7.500 mg. CO_2 and 2.470 mg. H_2O
$C_{10}H_{16}O_4$. Calculated. C 59.96, H 8.06
200.1   Found.   " 60.01, " 8.11

Levo-Mandelic Acid—100 gm. of *dl*-mandelic acid were resolved[4] with ephedrine.[5] After one crystallization of the salt from absolute alcohol, the crystals yielded 34 gm. of acid which had the following rotation.

$$[\alpha]_D^{25} = \frac{-13.8° \times 100}{2 \times 5.0} = -138°; \quad [M]_D^{25} = -210° \text{ (water)}$$

0.500 gm. of the acid was dissolved in 10 per cent sodium hydroxide to make 10 cc.

$$[\alpha]_D^{25} = \frac{-10.7° \times 100}{2 \times 5.72} = -93.5°; \quad [M]_D^{25} = -163° \text{ (sodium salt)}$$

4.535 mg. substance: 10.555 mg. CO_2 and 2.210 mg. H_2O

$C_8H_8O_3$. Calculated. C 63.13, H 5.30

152.1   Found.   " 63.47, " 5.45

Levo-2-Acetoxy-2-Phenylacetic Acid—22 gm. of mandelic acid, $[\alpha]_D^{25} = -138°$ (water), were acetylated as described previously.[6] A product which readily crystallized was obtained by dissolving the residue in ether, after the excess acetyl chloride had been distilled off, washing the solution with water until the last traces of acetyl chloride had been removed, and drying with drierite. After the ether was evaporated, the crystals were filtered. Yield 21 gm.

$$\frac{-38.5° \times 100}{4 \times 6.6} \quad -146°; \quad [M]_D^{25} = -283° \text{ (ether)}$$

4.894 mg. substance: 11.110 mg. CO_2 and 2.280 mg. H_2O

$C_{10}H_{10}O_4$. Calculated. C 61.83, H 5.19

194.1   Found.   " 61.90, " 5.21

Dextro-2-Acetoxy-2-Cyclohexylacetic Acid—3 gm. of acetylmandelic acid, $[\alpha]_D^{25} = -146°$ (ether), were dissolved in 20 cc. of glacial acetic acid and 0.5 gm. of Adams' catalyst was added. The suspension was shaken with hydrogen at a pressure of 3 atmospheres for 2 days at a temperature of 28°. The catalyst was filtered off

[4] Manske, R. H. F., and Johnson, T. B., *J. Am. Chem. Soc.*, **51**, 1906 (1929).

[5] We wish to thank Eli Lilly and Company for their kindness in supplying us with ephedrine.

[6] Gilman, H., Organic syntheses, New York, coll. **1**, 12 (1932).

and the filtrate was distilled.   The fraction boiling at 115–120°,
$p = 0.1$ mm., weighed 1.5 gm.

$$[\alpha]_D^{25} = \frac{+5.44° \times 100}{2 \times 15.4} = +17.7°; \quad [M]_D^{25} = +35.4° \text{ (benzene)}$$

5.402 mg. substance: 11.900 mg. CO_2 and 4.030 mg. H_2O

$C_{10}H_{16}O_4$.   Calculated.   C 59.96, H 8.06

200.1   Found.   " 60.07, " 8.34

STUDIES OF MULTIVALENT AMINO ACIDS AND PEPTIDES

VIII. THE SYNTHESIS OF BISANHYDRO-*l*-CYSTINYL-*l*-CYSTINE AND OTHER DIKETOPIPERAZINES OF CYSTINE

By JESSE P. GREENSTEIN

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Substances containing the R—SH group are readily oxidized to the corresponding R—S—S—R form. Molecules of the type $R(—SH)_2$, such as the homologous dimercaptobenzenes, yield on oxidation yellowish polymers of the general formula $(R—S)_x$ (15, 18–20).

The behavior of polythiol molecules is of particular interest owing to the profound changes in the properties of proteins due to changes in the state of the cystine residues contained in them. The oxidation-reduction equilibrium between the —SH and —S—S— forms is of importance in the physiological behavior of insulin (17), papain (7), urease (12, 16), and cathepsin (14). The appearance of a large number of —SH groups during the denaturation of proteins has been observed by several investigators (3, 4, 13). What form the reduced protein assumes on reoxidation of the thiol groups is at present unknown. The difficulties involved in a study of this sort are recognizably great. On the other hand, investigations of the problem of polythiol oxidation on simpler synthetic molecules may be more readily carried out and may illuminate the more complex behavior of the proteins. For this purpose the study of cysteinylcysteine derivatives was chosen and, as the first representative of this class of substances, the diketopiperazine, anhydro-*l*-cysteinyl-*l*-cysteine, was prepared.[1]

[1] While it is improbable that the diketopiperazine form occurs as such in the protein, it may serve as a peptide model in which the amino and carboxyl groups are tied off by self-condensation.

Anhydrocysteinylcysteine consists of a heterocyclic ring containing two methyl mercaptan groups substituted at the 3,6 positions. The two functional —SH groups are sufficiently separated to render intramolecular reaction improbable. On oxidation, it should yield a polymeric form in which the 3,6-dimethyl-2,5-diketopiperazine ring will form a repeating pattern, each element linked by disulfide bonds.

Dicarbobenzoxycystyl chloride (I) was coupled with 2 molecules of cysteine ethyl ester (II) in chloroform solution. The product, dicarbobenzoxycystyldicysteinyl ethyl ester (III) was reduced with phosphonium iodide according to the method of Harington and Mead (10). The resulting cysteinylcysteine ethyl ester hydroiodide (IV) was dissolved in ethanol saturated with ammonia at 0°. Condensation of the piperazine ring occurred and anhydro-l-cysteinyl-l-cysteine (V) appeared as a mass of well defined prismatic crystals. On purification, the product reduced close to the theoretical quantity of iodine. The oxidation of the dithiol proceeded with a slight excess of hydrogen peroxide in aqueous solution. The oxidized product crystallized in beautifully long colorless needles. Molecular weight determinations yielded values close to that of 408 for the dimeric form, bisanhydro-l-cystinyl-l-cystine (VI).[2]

$$\left[\substack{NHCbzo \\ | \\ -S-CH_2CHCOCl} \right]_2 + 2NH_2\substack{COOC_2H_5 \\ | \\ CHCH_2-SH} \longrightarrow$$

$$I \qquad\qquad\qquad II$$

$$\left[\substack{NHCbzo \quad COOC_2H_5 \\ |\qquad\quad | \\ -S-CH_2CH \quad CONHCHCH_2-SH} \right]_2 \longrightarrow$$

$$III$$

$$\substack{NH_2 \cdot HI \quad COOC_2H_5 \\ |\qquad\quad | \\ SH-CH_2CH \quad CONHCHCH_2-SH} \longrightarrow \substack{NHCO \\ SH-CH_2CH\diagup\quad\diagdown CHCH_2-SH \\ \diagdown\quad\diagup \\ CONH}$$

$$IV \qquad\qquad\qquad\qquad V$$

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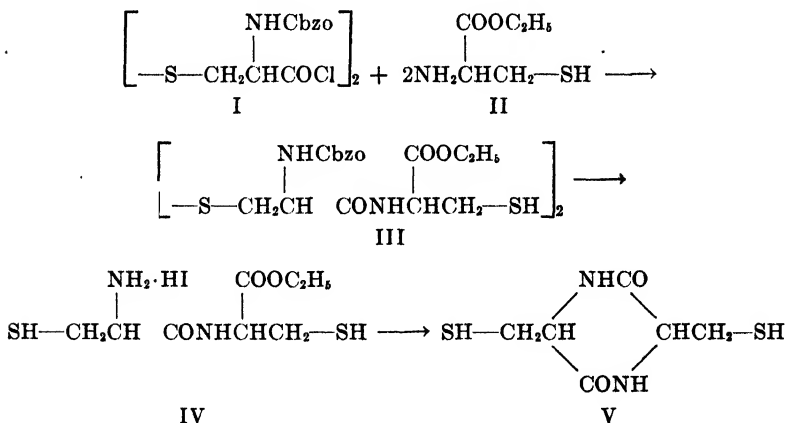
[2] Coghill (8) has suggested the formation of a polymeric anhydride of cystine during the spontaneous decomposition of cystine methyl ester. It was, however, not isolated.

```
                    NHCO
                  /       \
   S—CH₂CH            CHCH₂—S
   |           \       /           |
   |            CONH            |
   |                                |
   |            CONH            |
   |           /       \           |
   S—CH₂CH            CHCH₂—S
                  \       /
                   NHCO
```

VI
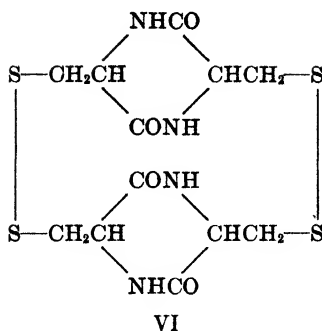
The possession of two functional methyl mercaptan groups substituted at the 3,6 carbon atoms of the heterocyclic ring did not, therefore, lead to an extended polymeric form but caused the molecule to assume the smallest possible cyclic configuration. It would be interesting to compare the behavior of anhydrocysteinylcysteine with that of the analogous ring compound, dithiolhydroquinone. Studies of the open chain dithiols are now in progress.

Several new diketopiperazines of cystine combined with trivalent amino acids were also prepared according to the general method outlined above. These included derivatives of cystine with the dicarboxylic amino acids; anhydro-*l*-cystyldi-*l*-γ-ethyl glutamate, anhydro-*l*-cystyldi-*l*-γ-methyl glutamate, and anhydro-*l*-cystyldi-*l*-β-ethyl aspartate. Anhydro-*l*-cystyldi-*l*-tyrosine was likewise synthesized. The latter substance contains two amino acids believed to be of significance in the physiological properties of insulin (1, 11). These anhydrides in contrast with the homogeneous diketopiperazines of cystine were all insoluble in water. The former three were soluble, however, in glacial acetic acid and in formamide. The tyrosine derivative on the other hand could be dissolved only in formamide. All the anhydrides described in this communication decompose on warming with alkali.[3]

EXPERIMENTAL

The diketopiperazines were prepared by condensing the peptide ester in alcoholic ammonia. The peptide ester was coupled by

[3] Other cyclic derivatives of cystine such as cystine phenylhydantoin (2) and cystine cyamidene (9) are likewise extremely labile toward alkali.

the Bergmann and Zervas carbobenzoxy technique (6), the benzylcarbonyl residue subsequently being removed by phosphonium iodide in acetic acid solution (10).

Dicarbobenzoxy-l-Cystyldi-l-Diethyl Glutamate—Dicarbobenzoxycystyl chloride prepared from 24 gm. of dicarbobenzoxycystine (6) was added at once to a chilled dry ether solution of excess diethyl glutamate. The mixture was shaken vigorously for an hour at room temperature, filtered, the residue washed with ether, then with ice water, and finally dried. The coupling product was crystallized by dissolving in hot ethanol and adding hot water to turbidity. On slow cooling, the substance appeared in long needles. Yield, 32 gm.; m.p., 145°.

$C_{40}H_{54}O_{14}N_4S_2$ (878.4). Calculated. N 6.4, S 7.3
                             Found.   " 6.9, " 7.3

Anhydro-l-Cystyldi-l-γ-Ethyl Glutamate—10 gm. of the coupling product were dissolved in 140 cc. of warm glacial acetic acid and treated with 10 gm. of pulverized phosphonium iodide. The mixture was placed in a water bath at 50° and a stream of dry hydrogen passed through. The reaction was complete after 3 hours. A thick yellow syrup remained when the solution was evaporated *in vacuo*. It was taken up in a little glacial acetic acid and treated with an excess of dry ether. The cysteinyldiethyl glutamate hydroiodide separated as a thick syrup. The ether was decanted and the syrup dissolved in alcohol and treated with dry NH_3 gas at 0° until saturation occurred. Crystallization of the anhydride began rapidly and was complete in 24 hours. It was filtered off, washed with alcohol, then with water, and allowed to dry in the air. The substance was then suspended in water to which some ammonia and a drop or two of $FeCl_3$ solution had been added, and the mixture was aerated for 48 hours. Crystallization was effected by dissolving the substance in hot glacial acetic acid and adding hot water to turbidity. On cooling, short needles separate. Yield, 4.1 gm.; m.p., 259°.

The diketopiperazine is soluble without decomposition in warm glacial acetic acid and in warm formamide. It dissolves in warm dilute alkali to give a yellow solution which on acidification evolves H_2S and yields a yellowish, CS_2-insoluble precipitate. The latter may be the analogue of that polymeric anhydride which Bergmann

and Stather isolated from the alkaline digestion products of anhydrodileucylcystine (5)

$C_{20}H_{30}O_8N_4S_2$ (518.3). Calculated. N 10.8, S 12.3
Found. " 10.9, " 11.9

Dicarbobenzoxy-l-Cystyldi-l-Dimethyl Glutamate—This was prepared exactly as the diethyl compound. Yield, 7.5 gm. from 8 gm. of dicarbobenzoxycystine; long needles from methanol-water; m.p., 139°.

$C_{36}H_{46}O_{14}N_4S_2$ (822.4). Calculated, N 6.8; found, N 6.9

Anhydro-l-Cystyldi-l-γ-Methyl Glutamate—Tufts of fine needles were obtained from dilute acetic acid; m.p., 258°.

$C_{18}H_{26}O_8N_4S_2$ (490.2). Calculated, N 11.4; found, N 11.5

Dicarbobenzoxy-l-Cystyldi-l-Diethyl Aspartate—The yield of coupling product from 8 gm. of dicarbobenzoxycystine was 6.5 gm.; long needles from ethanol-water; m.p., 145°.

$C_{38}H_{50}O_{14}N_4S_2$ (850.4). Calculated, N 6.6; found, N 7.0

Anhydro-l-Cystyldi-l-β-Ethyl Aspartate—Beautiful prisms were obtained from dilute acetic acid; m.p., 246°.

$C_{18}H_{26}O_8N_4S_2$ (490.1). Calculated, N 11.4; found, N 11.6

Dicarbobenzoxy-l-Cystyldi-l-Ethyl Tyrosinate—Dicarbobenzoxycystyl chloride from 4 gm. of dicarbobenzoxycystine was added to an excess of tyrosine ethyl ester in dry ethyl acetate, the mixture shaken for an hour, filtered, and the gelatinous coupling product washed with dilute HCl. After solution in hot ethanol, it was precipitated with hot water. A final crystallization from dilute acetic acid yielded a product melting at 168–175°. Yield, 3 gm.

$C_{44}H_{50}O_{12}N_4S_2$ (890.4). Calculated, N 6.3; found, N 6.3

Anhydro-l-Cystyldi-l-Tyrosine—Following the reduction, condensation, and oxidation procedures, the free anhydride was washed with dilute HCl, then with water, and finally dried in air. Yield, about 60 per cent of the theory. The substance was pure white in appearance, and insoluble in all non-aqueous solvents but

hot formamide. It was recrystallized by dissolving in an excess of hot formamide, chilling, and then treating with cold water. The diketopiperazine separated in tufts of very fine needles. M.p., 283° with brisk decomposition.

Although the material was soluble in cold dilute NaOH without apparent decomposition, attempts to precipitate it on immediate acidification were unsuccessful. Alteration of the molecule may have occurred during the very short space of time required completely to dissolve in alkali. Prolonged action of alkali results in visible decomposition.

$C_{24}H_{26}O_6N_4S_2$ (530.2). Calculated. N 10.6, S 12.1
Found.   " 10.7, " 11.8

Dicarbobenzoxy-l-Cystyldi-l-Ethyl Cysteinate (III)[4]—From 4.5 gm. of dicarbobenzoxycystine, the acid chloride was prepared with the aid of 4.1 gm. of PCl_5 in 80 cc. of dry ether. During filtration of the chloride, 4.5 gm. of cysteine ethyl ester hydrochloride were suspended in 75 cc. of dry chloroform and treated with 5 cc. of diethylamine. On shaking, the ester hydrochloride rapidly dissolved. The solution was chilled and the dicarbobenzoxycystyl chloride added rapidly with shaking. The clear solution was allowed to stand for 2 hours at room temperature and then filtered. The filtrate was shaken out with ice-cold dilute HCl, then twice with cold water, and finally dried for several hours over Na_2SO_4. The residue on evaporation *in vacuo* was a thick yellow oil. It was taken up in a little ethyl acetate and treated with an excess of petroleum ether. The gum which separated gradually hardened. M.p., 72–76°. It was used in the following procedure without purification.

$C_{32}H_{42}O_{10}N_4S_4$ (770). Calculated, N 7.2; found, N 7.2

Anhydro-l-Cysteinyl-l-Cysteine (V)—The raw coupling product dissolved in glacial acetic acid was treated as above with phosphonium iodide. The reduction was complete after 3 hours. Evaporation *in vacuo* yielded the dipeptide ester, cysteinylcysteine ethyl ester hydroiodide, as a thick yellow syrup. After the material was treated with ether, the syrup was taken up in ethanol and saturated as usual with NH_3 gas at 0°. Foci of crystallization

_____

[4] The roman numerals refer to formulæ in the introductory section.

began after several hours and crystallization was complete after 24 hours. The crystals were beautiful, well defined prisms. The substance was filtered off rapidly, washed with cold alcohol and ether, and dried *in vacuo*. Yield, 0.5 gm.

A 1 per cent solution in boiled water was saturated with H_2S gas and allowed to stand for 48 hours. It was then filtered, condensed *in vacuo*, and the clusters of large prisms formed were filtered off and dried rapidly. The substance crystallized from water with 0.5 molecule of water of crystallization. It began to sinter at 203° and melted with decomposition at 208°. It is soluble in water, alkali, mineral acids, and glacial acetic acid, not in organic solvents. The aqueous solution was slightly acid to litmus, gave a strong nitroprusside reaction, and yielded a flocculent precipitate with neutral lead acetate, silver nitrate, and mercuric sulfate. Treatment of the substance with alkaline plumbite rapidly yielded PbS at room temperature. With picric acid-K_2CO_3 it gave a very deep red color on warming. The ninhydrin reaction was negative. The material has a typical mercaptan odor.

0.096 gm. of the dried substance requires 11.9 cc. of 1 per cent iodine solution; found, 11.5 cc.

The compound was dried for analysis at 80° and 1 mm. pressure.[5]

$C_6H_{10}O_2N_2S_2$.   Calculated.   C 35.2, H 4.8, N 13.6, S 31.0
206.2   Found.   " 35.4, " 4.6, " 13.9, " 30.5
Calculated for 0.5 molecule H_2O, 4.2% (for the substance dried over H_2SO_4); found after drying at 80° at 1 mm., 4.0%
$[\alpha]_D^{23}$ for an 0.8% solution in water was −62.5°

Bisanhydro-l-Cystinyl-l-Cystine (VI)—0.35 gm. of the anhydro-*l*-cysteinyl-*l*-cysteine was dissolved in 40 cc. of water with warming. The mixture was chilled and 0.5 cc. of 29 per cent H_2O_2 solution was added. The mixture was placed in an ice bath. Within a few minutes it became turbid and an amorphous precipitate settled out. The latter was filtered off and discarded and the

[5] When the anhydride is dissolved in exactly 6 times the amount of cold concentrated HCl and allowed to stand at room temperature for 5 days, cysteinylcysteine hydrochloride crystallizes out in 50 per cent yield. M. p. 158°, $[\alpha]_D^{23}$ = +44.8°. On oxidation it yields crystalline cystinyl-cystine. These peptides will be described in a subsequent communication.

filtrate was allowed to evaporate slowly in a vacuum desiccator. Beautiful long, colorless needles, several mm. in length began to separate.   Yield, 0.25 gm.   The mercaptan odor had disappeared and the material failed to react with either iodine or nitroprusside. Heated in a capillary, it began to turn brown at 250° and gradually darkened up to 310° without melting.   The aqueous solution was neutral to litmus and the solid material was insoluble in cold ammonia solution in contrast to the behavior of the reduced product.   No precipitate formed with neutral lead acetate.   Addition of alkali to the lead solution, however, yielded PbS at room temperature within a short time.   The substance gave a deep red color with K_2CO_3-picric acid on warming.   It crystallizes from water with 0.5 molecule of water of crystallization.

For analysis the substance was dried at 80° and 1 mm. pressure.

$C_{12}H_{16}O_4N_4S_4$.   Calculated.   C 35.2, H 3.9, N 13.6, S 31.3
408.4   Found.   " 35.1, " 3.8, " 13.1, " 31.5
Calculated for 0.5 molecule H_2O, 2.2% (for the air-dried material); found after drying at 100° at 1 mm., 2.7%

$[\alpha]_D^{23}$ for a 0.4 per cent solution in water gave the extraordinarily high value of $+312.5°$.   The solution maintained this value over several hours standing at room temperature.

Molecular Weight Determinations—0.252 gm. of the carefully dried substance dissolved in 25.1 gm. of glacial acetic acid lowered the freezing point of the pure solvent by 0.105°; molecular weight calculated, 408; found, 370.

The diffusion method was likewise employed to estimate the molecular weight of the compound.   The rate of diffusion of the molecule through a sintered glass cell was determined and compared with the rates found for substances of known molecular weight.[6]   The time required, for example, for 1 cc. of 0.1 M glucose to diffuse was 18.5 hours; for the substance itself in 0.4 per cent solution 24.4 hours were required.   Comparison with values for other compounds of known molecular weight yielded an estimate close to 400 ± 20 per cent for the molecular weight of the compound and suggested the dimeric form (VI).

Hydrolysis of Bisanhydro-l-Cystinyl-l-Cystine—1 gm. of the sub-

stance was boiled for 5 hours with concentrated HCl under the reflux. The solution was filtered and concentrated *in vacuo*, the residue taken up in water, and the free cystine precipitated on neutralization with sodium acetate. The yield of dried cystine was 1.05 gm. or 89 per cent of the theory.

$C_6H_{12}O_4N_2S_2$ (240). Calculated, N 11.7; found, N 11.7

SUMMARY

1. Diketopiperazines of *l*-cystine combined with either glutamic acid, aspartic acid, or tyrosine have been prepared.

2. The preparation and properties of crystalline anhydro-*l*-cysteinyl-*l*-cysteine are described.

3. The oxidation with hydrogen peroxide of the dithiol diketopiperazine leads to the formation of the crystalline dimeric molecule, bisanhydro-*l*-cystinyl-*l*-cystine. Acid hydrolysis of the latter results in an 89 per cent recovery of cystine

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THE USE OF THORIUM NITRATE IN THE RAPID ASHING OF SERUM AND URINE

I. ADAPTED FOR SUBSEQUENT POTASSIUM DETERMINATIONS

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A method has been devised whereby serum or urine may be ashed in an electric muffle furnace in 20 minutes at 600° or in 10 minutes at 750° by the addition of a solution of thorium nitrate. 5 cc. of serum are ashed as quickly as 1 cc., but a proportionally larger amount of thorium must be added. The ash is dissolved in 0.1 per cent H_3PO_4, made up to the desired volume, mixed, centrifuged, and aliquots of the clear supernatant solution are used for the determination of potassium. Thorium is thought to form double salts with potassium, calcium, magnesium, and sodium which volatilize at a much higher temperature (1) than the individual salts, with no danger of loss of potassium even at 750°.[1] The greater part of the thorium in the ash is present as the insoluble oxide and the remainder is precipitated from an extract of the ash as the insoluble thorium phosphate. The solution, after elimination of the precipitate by centrifugation, is used in the determination of the bases and is thorium-free.[2] Thorium nitrate appears

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[1] Previously, material ashed for a potassium determination was heated no higher than 500° because of the probable danger of loss of potassium at a higher temperature. This may explain our failure to obtain satisfactory results by ashing for 3 hours or longer at 600°, as described by Hald (2).

[2] The serum or urine ash may be dissolved in 0.1 N HCl if desired, identical results being obtained whether H_3PO_4 or HCl is used in the extraction. Since H_3PO_4 eliminates all thorium, its use is preferred. When ashing has taken place at 750°, only small quantities of thorium are found in the HCl extract; at 600° larger quantities of thorium will appear in the extract. The presence of thorium *per se* does not interfere with the determination of potassium, but because of its insolubility in the platinic chloride-alcohol mixture, additional washing is necessary to prevent occlusion of the platinic chloride reagent.

to act as a catalyst in so far as the time required for the ashing of serum is shortened. A pure white ash is obtained in a maximum of 25 minutes when the dry $Th(NO_3)_4$-serum mixture is heated in an oven at 550–600°, whereas 10 to 12 hours at 500–600° are necessary to obtain a pure white ash from a serum-sulfuric acid mixture.

Reagent—

10 per cent thorium nitrate.[3, 4]

Ashing

Serum—Into a 50 cc. silica beaker are pipetted 1 cc. of serum, 1 cc. of 10 per cent thorium nitrate, and 2 drops of 4 N H_2SO_4.

Urine—A volume of urine or diluted urine is ashed which will give an amount of potassium within the range of the method suitable for its determination. Under these conditions 0.5 cc. of 10 per cent thorium nitrate is sufficient for the ashing. To insure acidity of the mixture and to expel any carbonate which would otherwise precipitate the thorium 0.5 cc. of 4 N H_2SO_4 is also added.

Foods—Various types of foods have also been ashed with the same procedure which is used for urine except that the sample was left in the oven for 30 minutes at 750° to complete the ashing. Satisfactory results were obtained in a fraction of the time formerly necessary to ash such material.

The solution is thoroughly mixed by carefully rotating the ·beaker. The beaker is placed on a steam bath and the contents evaporated to approximate dryness. This procedure takes no more than 20 to 30 minutes. The beaker is then placed on the outer door of an electric muffle furnace previously heated to 750°. It is allowed to stay there for a few minutes and is then moved in

[3] Obtained through the kindness of the Maywood Chemical Works, Maywood, New Jersey.

[4] The potassium and sodium contents of many samples of thorium nitrate were determined and were found to be so high as to give blanks which were too large for satisfactory use. The Maywood $Th(NO_3)_4$ was found to be a most satisfactory product. By using 0.1 per cent H_3PO_4 in dissolving the ash and precipitating out all of the thorium it was found that there was no potassium present in the thorium nitrate. If, however, 0.1 N HCl was used to dissolve the ash, some thorium was soluble. Usually it occluded enough reagent to necessitate the use of 0.04 cc. of 0.01 N $Na_2S_2O_3$ as a blank titration.

gradually. At no time is there any crackling or spattering. It takes no longer than 5 minutes to introduce the beaker into the furnace, at which time the muffle door is closed and the beaker is kept in the oven at 750° for 10 minutes. A snow-white ash is obtained.

Solution—To the ash is added 1.0 cc. of 0.1 per cent H_3PO_4. The ash is pulverized by means of a glass rod and mixed thoroughly. Then 5.0 cc. of 0.1 per cent H_3PO_4 are added and the contents of the beaker are again carefully mixed. The contents are then transferred to a clean, dry, 15 cc. centrifuge tube, centrifuged, and the clear supernatant fluid is decanted. A 5 cc. aliquot of the supernatant fluid is used for the potassium determination. A factor of 1.2 is introduced into the calculations, as 5 cc. of this solution represent 0.83 cc. of serum.

Method of Analysis

Potassium—5 cc. of dissolved ash, equivalent to 0.83 cc. of serum, are taken to dryness on the steam bath in a 50 cc. Pyrex beaker and potassium is precipitated as the potassium chloroplatinate according to the method of Shohl and Bennett (3) but with the modification of Hald (2), in which absolute alcohol saturated with potassium chloroplatinate is used in precipitating the potassium. All results for potassium reported in this paper were determined by the titrimetric procedure with 0.01 N $Na_2S_2O_3$ as described by Shohl and Bennett.

Results

Standard solutions of K_2SO_4 have been added to serum and urine before ashing with $Th(NO_3)_4$ and have been recovered with an error of ±2.6 per cent or less (Table I).

Several samples of serum and urine were ashed simultaneously with the aid of thorium nitrate and also by Neumann's wet ash method (4) with a mixture of concentrated H_2SO_4 and HNO_3. The wet ashing took place in a large Pyrex test-tube over a free flame. The excess sulfuric acid and the ammonia were driven off in an electric muffle furnace at 500°. The difference in the potassium results was ±2 per cent between the $Th(NO_3)_4$ ash and the Neumann ash methods. This is within the limit of error of the method used in determining potassium and shows that there is no

TABLE I

Recovery of Added K_2SO_4 Standard Solution

In each case 0.001 part of the amount reported was ashed and analyzed.

Substance analyzed	Potassium found	Potassium added*	Potassium after addition of standard		Variation from theoretical
			Theoretical	Found	
	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	per cent
Human serum.................	4.57	5.13	9.70	9.74	+0.4
" "	3.67	5.13	8.80	8.80	0.0
" " "	4.24	10.14	14.38	14.00	−2.6
" "	4.45	10.14	14.59	14.59	0.0
Hemolyzed serum.............	6.24	5.13	11.37	11.33	−0.4
Dog urine.................	9.71	5.13	14.84	14.78	−0.4
" "	11.99	5.13	17.12	17.55	+2.5
" "	14.63	5.13	19.76	19.65	−0.6
Human "	11.19	10.02	21.21	21.40	+0.9
Dog "	9.06	5.13	14.19	14.40	+1.5

* The amount of potassium which was added before ashing to the same amount of material, the analysis of which is found in the preceding column.

TABLE II

Potassium Recovered by Two Different Methods of Ashing

All results are expressed as milli-equivalents of potassium per liter. In each case 0.001 part of the amount reported was ashed and analyzed.

Serum analyzed		Urine analyzed	
$Th(NO_3)_4$ ash	Neumann wet ash	$Th(NO_3)_4$ ash	Neumann wet ash
5.06	5.07	4.19	4.22
4.62	4.65	13.81	13.83
4.43	4.55	13.94	13.95
4.62	4.75	10.46	10.45
4.11	4.12	11.23	11.19
5.25	5.23	5.47*	5.42

* This result was obtained on the whole blood of a dog. 0.5 cc. of 4 N H_2SO_4 and 1 cc. of 10 per cent $Th(NO_3)_4$ were added before ashing and the sample was left in the oven for 30 minutes at 750°.

adsorption of the base by the rare earth. These results are shown in Table II.

SUMMARY

A method is presented for the rapid ashing of serum, urine, or similar biological material, by the use of a salt of the rare earth thorium, in a maximum of 15 minutes at 750° or in 25 minutes at 600°.

The amounts of potassium found with the thorium ashing method agree with those found with the Neumann wet ash technique. Theoretical increments of potassium were found when pure standard K_2SO_4 solutions were added to serum or urine before ashing.

It is probable that this method of ashing may be adapted to the determination of sodium. By the addition of dry $Ca(OH)_2$ to the solution of the ash the phosphate is readily eliminated.

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A CONVENIENT TYPE OF TONOMETER FOR THE EQUILIBRATION OF BLOOD

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For determining the oxygen and carbon dioxide capacities of blood in equilibrium with known pressures of those gases a compact and convenient tonometer combined with a blood pipette has been described by Barcroft.[1]   We have found that the structure of the tonometer can be modified to make it more compact and sturdy without sacrifice of accuracy.

The construction of the combined tonometer and pipette is shown in Fig. 1.   The capacity of the ordinary tonometer is about 350 ml., but the dimensions D and E (Fig. 1) are mainly determined by the apparatus for equilibration and the quantity of blood which is to be used.   The mouth of the vessel is ground at A to receive the correspondingly ground enlargement of the pipette.   This enlargement is bored at B (Fig. 2) to provide an opening through the side tube by means of which the gas sample is removed.   In order that blood may not enter the gas sampling tube, a roll (F) is provided at the free end of the ground pipette enlargement.   The neck of the tonometer and pipette enlargement should both be made as strong as possible.   The pipette may have one or two bulbs according to whether duplicate analyses are desired.   The length C of the pipette must be sufficient to reach the bottom of the cup in the Van Slyke blood analyzer. A convenient bore for the capillary of the pipette is from 1.5 to 1.8 mm.   If the bore is slightly enlarged at the junctions with the bore of the stop-cock, to coincide with a stop-cock bore of about 2.2 mm., the blood is more likely to move past the inevitable line of grease at the junction of capillary and pipette without trapping bubbles.

[1] Barcroft, J., *J. Physiol.*, **80**, 388 (1934).

For use, blood is introduced into the tonometer.   If the tonometer is shaped to drain well into the pointed end, 3.5 mls of blood will fill a pipette containing two 1 ml. samples.   With smaller dimensions 1 ml. of blood will regularly fill a 0.5 ml. pipette.
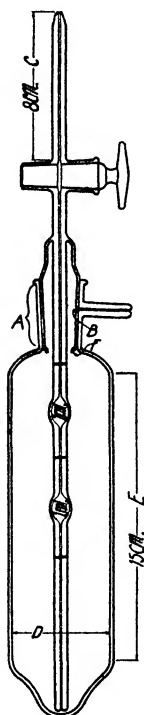
FIG. 1. Section through assembled tonometer and pipette

FIG. 2. Cross-section through ground pipette-tonometer connection at *B* (Fig. 1).

The pipette is inserted and a known gas mixture is used to flush the tonometer or it is first exhausted to 0.5 atmosphere, flushed from five to ten times with moist nitrogen, and then filled with gas components according to manometric measurement.

Both apertures to the tonometer are then closed and it is ready to be rotated in the water bath for equilibration at a higher temperature than that which prevailed during filling. The increased pressure which results from equilibrating at a higher temperature is utilized, after equilibration is complete, to fill the pipette. In order to do so the tonometer is held vertically in the water bath to allow the blood to drain into the conical end. After the blood has collected about the tip of the pipette, the stop-cock at the other end of the pipette is very slowly opened, and the blood passes up to fill the pipette.

It is the utilization of the pressure produced by the higher temperature of equilibration which determines the least volume of the tonometer for a given volume of blood. If the filling occurs at 20° and equilibration at 37°, if a ml. of blood are required to fill the pipette, and if the top of the pipette is 15 cm. above the bottom, then the volume of the tonometer, V, must bear at least this relation to a:

$$\frac{17}{310} - \frac{15}{76 \times 13.5} = \frac{a}{V}, \text{ or } V = 25a$$

Since for the purpose of equilibration the gas volume is usually about 100 times as great as the volume of blood, the pressure produced by a 15° change in temperature is quite sufficient to fill the pipette. For special volume relations, or when the temperature of equilibration is low, consideration must be given to these dimensions. In the case of equilibrations at lower temperature, it may be necessary to fill the tonometer with gas in a water bath at lower temperature—say at 10° for equilibration at 20°.

As soon as the pipette is filled, the stop-cock is closed, and with the tonometer still in the water bath, the sample of gas for analysis is withdrawn through the side opening into a gas-handling pipette. The pipette containing the blood samples can then be removed and its contents run directly into the Van Slyke apparatus for analysis.

It is simple and accurate to allow for the slight elevation of pressure of the gas during equilibration. After equilibration the blood and gas are immediately and easily separated for analysis. The apparatus is rugged and cheaper to construct than an

ordinary blood pipette and separate tonometer, and it has been found to be practical for the use of students as well as for research.

The tonometers may be obtained from Mr. R. W. Chapple, University of Toronto, Toronto, or from Mr. J. D. Graham, University of Pennsylvania, Philadelphia.

ARE THE PHOSPHATASES OF BONE, KIDNEY, IN-
TESTINE, AND SERUM IDENTICAL?

THE USE OF BILE ACIDS IN THEIR DIFFERENTIATION

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The demonstration of increased concentrations of phosphatase[1]
in the serum in physiological processes and pathological conditions
has been pursued intensively during the past few years.   Illustra-
tive of many such studies are those of Kay (1), A. Bodansky and
Jaffe (2), Woodard, Twombly, and Coley (3) on bone diseases, of
Roberts (4) and Rothman, Meranze, and Meranze (5) on various
types of jaundice, and of Gutman, Tyson, and Gutman (6) on
hyperparathyroidism, Paget's disease, multiple myeloma, and
neoplastic diseases of bone.   Franseen and McLean (7) have
compared the phosphatase activity of plasma and bone tissue in
various tumors of the bone.   Investigation has also been made of
the level of serum phosphatase during digestion (8), in experi-
mental bile duct ligation (9, 10), and after removal of various
organs (11).

It has been generally assumed in such work and proof has been
offered by Kay (12, 13) that the phosphatases of the different
tissues and of serum are probably identical, and attempts at
elucidating the nature and source of serum phosphatase in health
and disease have been made by several investigators.   Thus Kay
(1) has offered the explanations that the high plasma phosphatase
in bone diseases comes from the bones either as an overproduction
in an attempted compensation for the lesion or because of squeez-

[1] The term "phosphatase" is used in this paper to denote the enzyme
acting optimally at a pH of about 9.0 on the monophosphoric esters and
on hexosediphosphoric acid.

ing out from the cells of the injured bone under mechanical stress. A. Bodansky and Jaffe (2) and A. Bodansky (8) consider the increase in bone disease of certain types an expression of the "specific reactivity of bone" or its capacity for cellular activities; non-osseous origins of serum phosphatase in the normal are predicated because of the finding of hyperphosphatasemias after carbohydrate ingestion in young dogs and abundant feeding in new born puppies.   A more direct experimental approach has been recently submitted by Armstrong and Banting (11) who showed that in the dog extirpation of the intestines, kidneys, spleen, pancreas, liver, testes, or epididymes does not lower the serum phosphatase and may, indeed, raise it.   Bone is therefore considered as the sole source of serum phosphatase.

Preliminary to the investigation of such source and of the mechanism of the various hyperphosphatasemias is the necessity for determining whether the phosphatases of the tissues and of the serum are indeed identical or, if not, the manner in which they differ and the means by which they may be distinguished.

The present paper describes attempts to answer some portion of these questions by studying the pattern of hydrolysis by the different tissue phosphatases of several phosphoric esters, and the hydrolysis of sodium β-glycerophosphate in the presence of different types of retardants, principally and usefully, the bile acids. Since the bone, kidney, and intestine have been considered to contain the highest concentrations of phosphatase (12),[2] the present investigation has begun with the study of the phosphatases of these tissues from several species, including man.   It is shown that the bile acids retard the hydrolysis of sodium β-glycerophosphate by bone and kidney phosphatases but do not affect that by the intestinal phosphatases.   The applicability of these findings to the study of the source and nature of serum phosphatase, in health and in disease, is then considered.

<div align="center">EXPERIMENTAL</div>

Preparations of Phosphatase—In the designation of the preparations used in the course of the present work, the first capital letter denotes the species from which the tissue was obtained; a second

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[2] Recently Folley and Kay (14) have found the mammary gland of guinea pig to contain comparable concentrations of phosphatase.

small letter is added, if necessary: C represents cattle, Ca cat, R rat, M man, Rb rabbit. The second capital letter represents the tissue: B represents bone, K kidney, I intestine; and the third capital letter merely represents stock batches of phosphatase made up at different times. The small letter, d, immediately following the hyphen designates dialysis, usually overnight against a 20-fold volume of distilled water, changed once, as has previously been described (15). A small letter, p, following d, designates that dialysis was continued until a precipitate appeared, and that a solution was made from this precipitate with the aid of a little dilute sodium hydroxide. The capital letter, P_r following immediately after the hyphen designates that the preparation was made as a powder by the method of Albers and Albers (16), to be described, and d following P, in this case, indicates dialysis of the solution made from this powder. Numbers following d represent different batches of the stock phosphatase extracts which were taken for dialysis.

Most of the preparations were obtained, as previously described (15, 17), by extraction and 2 to 3 day autolysis of the tissue at room temperature; 20 cc. of distilled water and 1 cc. of toluene were used per gm. of tissue. Human tissues were obtained at the autopsy table. The kidney phosphatases, Preparations CKA-Pd, CKB-Pd, and MKA-Pd, were prepared by the method of Albers and Albers (16). This consists in a 4 to 5 day autolysis of the tissue in a mixture of toluene, ethyl acetate, and 50 per cent ethyl alcohol. After completion of autolysis, ethyl alcohol is added to a concentration of 65 per cent; the precipitate contains the phosphatase, is centrifuged, and may be dried with alcohol and ether. The dried preparation maintains its activity well. In the present studies, the precipitate was dissolved with the aid of a little dilute sodium hydroxide. The activity of such dialyzed preparations remained constant for weeks. The serum phosphatases were obtained from rabbit, cat, and man. The serum from two subjects with Paget's disease (osteitis deformans) were used for the illustration of the reaction of a pathological serum. The serum was not subjected to any purification or dialysis, but was usually diluted with water or saline and used in a concentration of 12.5 per cent by volume of the hydrolysis mixture. There was no attempt to use the enzyme preparations at a given time after extraction; it

was considered desirable that whatever distinguishing reactions they might show should be independent of this time factor.

Substrates and Retardants—All substrates were used in a concentration of 0.0127 M, calculated on the basis of the phosphorus that could be liberated as inorganic phosphate. The sodium β-glycerophosphate was the Eastman Kodak product. Sodium hexosediphosphate was prepared from the calcium salt, candiolin (Winthrop Chemical Company), by treatment with a submaximal amount of sodium oxalate to avoid subsequently the possible retardant effect of the oxalate ion; the resulting solution contained, per cc. of hydrolysis mixture, 0.025 mg. of calcium and 0.045 mg. of phosphorus as inorganic phosphate. The propylphosphoric acid was prepared as the barium salt and the benzylphosphoric acid as the potassium salt, according to Asakawa (18). The barium salt of propylphosphoric acid was dissolved in minimal hydrochloric acid, and the barium precipitated with sodium sulfate.

Conditions of experimentation necessarily limited the use of certain retardants. Thus mercuric chloride was employed in concentrations below 0.00125 M to avoid the formation of mercuric hydroxide at the pH of the hydrolysis mixture. The use of the cinchona alkaloids was limited either because of their low solubility at the alkaline pH of the hydrolysis mixture or because, in the subsequent Fiske-Subbarow method (19) for the determination of phosphorus, they were precipitable as phosphomolybdic ·salts (20) and thus capable of distorting the determination of phosphorus.

Sodium taurocholate and sodium glycocholate were Pfanstiehl products; sodium desoxycholate, dehydrocholic acid, and glycocholic acid were Riedel-de Haen products.[3] The acids were made up as the sodium salts by solution in an equivalent amount of dilute sodium hydroxide; the sodium dehydrocholate was made up just before use. In the optimal pH range (9.0 to 9.2) for the hydrolysis, the bile acids were present as the salts; reference in the text is made interchangeably to acid or salt.

The trichloroacetic acid in which the hydrolysis samples were introduced in order to stop the reaction and precipitate the protein

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[3] I am obliged to the Winthrop Chemical Company for the candiolin used in these experiments, to Riedel-de Haen, Incorporated, for the dehydrocholic acid, and to Professor W. T. Dawson for the cinchona alkaloids.

was found to precipitate all the bile acids used with the exception of cholic acid. It was noted that sodium cholate, not precipitated by the trichloroacetic acid, was precipitated in the course of the subsequent Fiske-Subbarow determination, together with molybdate and phosphate, thus giving low and incorrect values for the latter. For this reason, the use of cholic acid was avoided in the present studies.

The possibility of precipitation of bile acids by magnesium ion was noted by Schmidt and Merrill (21) and originally by Tengström (22). Of the bile acids used in the present study, only desoxycholic was precipitated by the optimal concentration of magnesium ion (0.009 M); for this reason the sodium desoxycholate was used in a somewhat lower concentration (0.00125 M) in the hydrolysis mixture, than the other bile acids (0.00625 M). The bile acids, as well as other retardants, were tested under conditions corresponding to those of the experiments to insure lack of effect on the determination, by the Fiske-Subbarow method, of phosphorus.

Determination of Activity of Phosphatases—In previous papers (15, 17) it was shown that α-amino acids in very low concentrations increased the activity of phosphatase by preventing its inactivation during the course of the reaction. Optimal prevention of inactivation occurred at a concentration of 0.00625 M for glycine. Magnesium ion was found to increase the velocity with which the reaction started; in agreement with previous workers (12), the optimal effect occurred at concentrations of 0.001 to 0.01 M. In the presence of optimal concentrations of both magnesium and glycine, a direct proportionality was established between reaction velocity and concentration of phosphatase and this was taken to indicate that, under these conditions, the concentration of active enzyme was the same as that of the apparent concentration. In the present study, therefore, the activity has been determined in the presence of 0.00625 M glycine and a concentration of magnesium in the optimal range, usually 0.009 M. It was determined, on bone and intestinal phosphatase preparations, that these concentrations were optimal for the hydrolysis in the presence of bile acids.

The desirability of comparing enzyme activity at optimal pH in studies of the effect of variation of substrate or enzyme concen-

tration, or of the presence of retardants, is generally recognized. The precise range of pH necessary for the optimal action of phosphatase on the substrates considered in the present work is subject to some disagreement owing to the fact that different investigators have used enzyme extracts prepared in different ways, different buffers, and different methods of measuring the extent of enzyme action. However, in general, such work, especially that done more recently (12, 18), indicates that the pH range for optimal action is narrow (9.0 to 9.2) and the slopes of the pH-activity curve on either side of the optimum steep.

For these reasons it was necessary, as has previously been stated (15, 17), to insure in each case that the determination of the phosphatase activity was indeed conducted at the optimal pH. For each determination a series of hydrolyses, constituting in effect a very closely spaced pH-activity curve in and about the optimal range, was run. The hydrolysis showing optimal action was chosen to represent the activity. Numerous pH determinations, mostly colorimetric, were made and agreed with the observations of previous workers that optimal activity was in the region pH 9.0 to 9.2. The presence of bile salts or other retardants, in the concentrations employed, did not alter this optimal range. This method, though somewhat laborious, has been used previously in successfully eliciting precise relationships between the activity and concentration of phosphatase (15, 17).

The technique of conducting the determination of activity was as follows: To each of four test-tubes, 1 cc. of 0.1016 M sodium β-glycerophosphate (or an appropriate volume of the solution of another substrate), 0.4 cc. of 10 per cent sodium diethylbarbiturate, 0.1 cc. of 0.5 M glycine, and 0.05 cc. of 1.45 M magnesium chloride were added. The amount of sodium hydroxide was graded in the four tubes, usually from 0.0 to 0.3 cc. of 0.2 N sodium hydroxide. Distilled water was then added so that when the phosphatase extract was introduced the total volume amounted to 8 cc. In those cases in which retardants were used, appropriate volumes of solutions of these were added. The concentration of phosphatase was expressed as volumes per cent of extract present in the hydrolysis mixture.

The solution to be hydrolyzed and the enzyme were brought to temperature in a thermostat maintained at 25° ± 0.05° (or any other temperature as given in the text). The phosphatase extract,

usually 1 cc., was added at a given time to the tube containing the hydrolysis mixture. The tube was immediately removed from the thermostat for a few seconds, and the contents were thoroughly mixed. Cooling was negligible.

At given time intervals samples of 1 cc. were withdrawn and added to a measured volume of trichloroacetic acid, filtered when there was a precipitate of protein or bile acid, and an aliquot taken for the determination of inorganic phosphate. The amount liberated per cc. of hydrolysis mixture was calculated. The amount of inorganic phosphate present at 0 time was calculated from the determinations of the content of inorganic phosphate in the substrate, enzyme extract, etc., if these contained any. The time course of the reaction was plotted. The reciprocal of the time in minutes necessary for the liberation of 0.05 mg. of phos-

TABLE I

Early Course of Hydrolysis Showing Inorganic Phosphate Liberated per Cc. of Hydrolysis Mixture with Optimal Activity at pH 9.1

Time	Concentration of P as inorganic phosphate	P liberated as inorganic phosphate
min.	*mg.*	*mg.*
0	0.0007	
33	0.0370	0.0363
46.5	0.0497	0.0490
59	0.0612	0.0605

phorus as inorganic phosphate per cc. of hydrolysis mixture was designated as Q and represented the activity of the enzyme.

A typical determination is illustrated by the following protocol. Four test-tubes containing solutions, made up as just described, were brought to 25°. At a given time, 1 cc. of bone phosphatase, Preparation CaBA-d3, similarly brought to 25°, was added to each tube, and the contents immediately mixed. Samples were taken at 33, 46.5, and 59 minutes. Since there was no protein precipitate when the samples were introduced into the trichloroacetic acid, it was not necessary to filter the latter. The inorganic phosphate was determined by the Fiske–Subbarow method (19) in a final volume of 10 cc., and calculated per cc. of hydrolysis mixture. The readings for different points in the hydrolysis showing optimal activity, at pH 9.1, are given in Table I. The phosphorus liberated was plotted against the time; the abscissa corresponding to

the ordinate 0.0500 mg. was 47.6 minutes. Q, the reciprocal, was 0.0211.

TABLE II

Rate of Hydrolysis of Phosphoric Monoesters and of Hexosediphosphoric Acid by Tissue Phosphatases

Temperature, 25°; concentration of phosphatase extract, 12.5 per cent by volume of the hydrolysis mixture, except for Preparation RbIA-d, 50 per cent; concentration of substrate, 0.0127 M, on the basis of phosphorus liberated as inorganic phosphate. Activity is expressed as the reciprocal of time in minutes necessary for liberation of 0.05 mg. of phosphorus as inorganic phosphate per cc. of hydrolysis mixture, and also relative to the rate of action when sodium β-glycerophosphate is used as substrate. All determinations are in the optimal pH range, 9.0 to 9.2; at optimal concentrations of glycine, 0.00625 M, and of magnesium, 0.009 M.

Preparation	Sodium β-glycero-phosphate	Potassium benzyl-phosphate		Sodium hexosedi-phosphate		Sodium propyl-phosphate	
	$Q_{0.05}$	$Q_{0.05}$	Relative activity	$Q_{0.05}$	Relative activity	$Q_{0.05}$	Relative activity
Bone phosphatases							
			per cent		per cent		per cent
CBH-dp	0.0083	0.0071	85	0.0043	52	0.0007	8
CBJ-d	0.0092	0.0067	73	0.0051	55	0.0007	7
CaBA-d	0.0203	0.0060	30	0.0140	70		
Kidney phosphatases							
CKA-Pd	0.0463	0.0468	101	0.0378	81	0.0044	10
RKJ-d	0.0120			0.0087	73		
RKH-d	0.0017			0.0011	65		
Intestinal phosphatases							
RIH-d3	0.0333	0.0285	86	0.0318	96	0.0017	5
RbIA-d	0.0127*	0.0197	155	0.0149	117	0.0013	10
CaIA-d	0.0145	0.0105	72	0.0083	57	0.0006	4

* This value was calculated from the activities for 12.5 per cent and 75 per cent of this preparation; proportionality between concentration of enzyme and activity for this preparation was previously reported (15).

Results

Action of Phosphatases on Phosphoric Esters—In Table II are shown the activities, $Q_{0.05}$, of the tissue phosphatases on the vari-

ous substrates. When the reaction velocity, $Q_{0.05}$, for the action on sodium β-glycerophosphate was taken as 100, and the reaction velocities for the hydrolysis of other substrates were compared to it, it was seen that the phosphatases of a given tissue from different species did not exhibit a constant pattern of relative actions. To illustrate, a cattle bone phosphatase, Preparation CBH-dp, showed a relative activity of 85 for the benzylphosphate and 52 for the hexosediphosphate; a cat bone phosphatase, Preparation CaBA-d, showed for these two substrates relative reaction velocities of 30 and 70, respectively. The other tissue phosphatases showed similar variations, and these variations were such as to make it impossible to differentiate an intestinal from a kidney or a bone phosphatase.

Effect of Cinchona Alkaloids, and of Mercuric Chloride on Activity of Phosphatases—Rona and his coworkers (23, 24) found that quinine and some related alkaloids decreased the activity of the invertases and of the lipases, the latter to varying degrees, depending on their source. As was mentioned earlier in this paper, the concentration in which the cinchona alkaloids could be tested for their effect on the phosphatases was limited by their solubility at the optimal pH (9.0 to 9.2) of the reaction and by the possibility of formation of a phosphomolybdate precipitate in the subsequent Fiske-Subbarow determination for phosphorus.

Rona and Reinicke (24) found that 7×10^{-6} M quinine retarded the activity of human serum lipase 50 per cent. A few preliminary experiments showed that neither quinine nor cinchonine in a concentration of 12.5×10^{-6} M nor quitenidine in the higher concentration of 19×10^{-5} M had any definite effect on the hydrolysis of sodium β-glycerophosphate by bone, kidney, or intestinal phosphatases.

Mercuric chloride has been found to decrease the activity of ptyalin, pepsin, invertase, and catalase (25–27). In the present study it was found that 0.00125 M mercuric chloride, within the range of the concentrations reported retardant for the other enzymes, decreased the activity of the phosphatases. These decreases were, however, not marked and did not serve to differentiate the phosphatases from different tissues. Thus, $Q_{0.05}$ for bone phosphatase, Preparation CBJ-d, decreased from 0.0092 to 0.0075 or 19 per cent, that for kidney phosphatase, Preparation

CKB-Pd, from 0.071 to 0.065 or 9 per cent, and that for intestinal phosphatase, Preparation CaIB-d, from 0.119 to 0.010, or 10 per cent.

Effect of Bile Acids on Activity of Bone, Kidney, and Intestinal Phosphatases—The salts of the bile acids have been shown to increase the activity of lipases (28, 29) and of trypsin in the acid range (30), and to decrease the activity of trypsin in the alkaline range (30). Sodium cholate has been reported to decrease the activity of kidney, bone, and liver phosphatases (31, 32) of dialyzed kidney and liver lecithinases (33), and to increase the activity of these undialyzed tissue lecithinases. As was mentioned earlier in this paper, it was not found possible to study the effect of sodium cholate on the activity of the phosphatases, since this bile acid did not precipitate in the trichloroacetic acid used to stop the hydrolysis and, as appeared from experiments with known amounts of phosphorus, precipitated together with phosphate and molybdate in the subsequent Fiske-Subbarow procedure.[4]

Table III shows the effect of the sodium salts of taurocholic, glycocholic, desoxycholic, and dehydrocholic acids on the rate of hydrolysis of sodium β-glycerophosphate by bone, kidney, and intestinal phosphatases. The reaction velocities in the presence of the bile acids are expressed as percentages of the original reaction velocity. Thus for the bone phosphatase, Preparation CaBA-d3, the value for $Q_{0.05}$ was 0.0211, 0.0198 in duplicate determinations; in the presence of 0.00625 M sodium taurocholate, the values for $Q_{0.05}$ were 0.0114, 0.0105, 0.0104, and the average of these, 53 per cent of the reaction velocity in the absence of bile acid. $Q_{0.05}$ for the intestinal phosphatase, Preparation CaIB-d2, was 0.0126. In the presence of 0.00625 M sodium taurocholate, values for $Q_{0.05}$ were 0.0127, 0.0121, and the average of these, 98 per cent of the original reaction velocity. In general, the error in the determination of Q, under the conditions of the present experiments, can be placed at less than 5 per cent.

The results show a clear cut differentiation between the effect of the bile acids on the activity of bone and kidney phosphatases,

[4] Takata (31) determined inorganic phosphate by precipitation from the trichloroacetic acid filtrate with magnesia mixture, conversion of the magnesium ammonium phosphate into ammonium phosphomolybdate, and titration.

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TABLE III

Effect of Bile Acids on Activity of Bone, Kidney, and Intestinal Phosphatases

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Temperature, 25°, except for Preparations CaBA-d2 and MIA-d, 28°; concentration of enzyme, 12.5 per cent by volume, except for Preparations CaBA-d2 and RbIA-d, 50 per cent; for Preparation MIA-d, 37.5 per cent, for Preparation CKB-Pd, 6.25 per cent, and for Preparation MIC-d, 25 per cent. The concentration of bile acid as the sodium salt is 0.00625 M, except for sodium desoxycholate, 0.00125 M. The activity in the presence of bile acid is expressed in per cent of the original activity of the phosphatase; all determinations at optimal pH, 9.0 to 9.2; and optimal concentrations of glycine, 0.00625 M, and magnesium, 0.009 M. Concentration of sodium β-glycerophosphate, 0.0127 M.

Preparation	Activity $Q_{0.05}$	Relative activity in presence of			
		Sodium taurocholate	Sodium glycocholate	Sodium desoxycholate	Sodium dehydro-cholate
Bone phosphatases					
		per cent	*per cent*	*per cent*	*per cent*
CBJ-d	0.0092	47	40	67	66
CBH-dp	0.0083	51			64
CaBA-d	0.0203	56	43	70	63
CaBA-d3	0.0205	53	43		71
CaBA-d2	0.0940	55			
Kidney phosphatases					
CKA-Pd	0.0463	47		73	63
CKB-Pd	0.0355		43	75	
RKJ-d	0.0120	58			
RKH-d	0.0017	50			
CaKA-d	0.0047				51
MKA-Pd	0.0295	52	43	65	57
Intestinal phosphatases					
RIH-d3	0.0333	100	96	100	98
RbIA-d	0.0127	97			
CaIA-d	0.0145	98			
CaIB-d	0.0119	96			98
CaIA-d2	0.0114		99	100	
CaIB-d2	0.0126	98	107	96	105
MIA-d	0.0175	103			
MIC-d	0.0073	104	102	102	84
MID-d	0.0227	100	103	105	

on the one hand, and on that of the intestinal phosphatases, on the other. The rate of hydrolysis of sodium β-glycerophosphate by the intestinal phosphatases is not affected by the presence of taurocholic, glycocholic, desoxycholic, nor, except for some slight retardation of the action of Preparation MIC-d, by dehydrocholic acid. In contrast, the retardation of the activity of the kidney and bone phosphatases by the same concentrations of bile acids is

TABLE IV

Effect of Inactivated Phosphatase Tissue Extracts on Retardation of Phosphatase Activity by Taurocholic Acid

Temperature, 25°; concentration of substrate, sodium β-glycerophosphate, 0.0127 M; concentration of taurocholic acid as sodium taurocholate, 0.00625 M; optimal pH, 9.0 to 9.2; and optimal concentrations of glycine, 0.00625 M, and magnesium, 0.009 M.

Phosphatase preparation	Inactivated phosphatase preparation	$Q_{0.05}$	
		No sodium taurocholate added	In presence of sodium taurocholate
Intestinal preparations, active			
6.25% cat intestinal, CaIB-d	6.25% cat bone, CaBA-d	0.0059	0 0059
12.5% human intestinal, MID-d ·	12.5% cat bone, CaBA-d3	0.0194	0.0207
11.1% human intestinal, MIB-d	11.1% human kidney, MKA-Pd	0.0047	0.0051
Bone preparations, active			
12.5% cat bone, CaBA-d3	12.5% human intestinal, MID-d	0.0198	0.0110
12.5% cat bone, CaBA-d3	12.5% cat intestinal, CaIB-d2	0.0198	0.0093

marked: a decrease to about 50 per cent in the presence of taurocholic acid, to about 45 per cent for glycocholic acid, and to about 65 per cent for desoxycholic and dehydrocholic acids. The existence of retardation of phosphatase activity by bile acids is thus characteristic of the tissue source of the phosphatase and, to the extent here investigated, independent of the species.

The results of Table IV and, as will be pointed out later, also those of Tables VI and VII, indicate that the effect of the bile

acids is probably not dependent on any substance in the tissue extracts other than the active enzymic component. Thus the addition of 12.5 per cent of the heat-inactivated cat intestinal phosphatase, Preparation CaIB-d2, did not alter, beyond experimental variation, the extent of retardation of the activity of the cat bone phosphatase, Preparation CaBA-d3, by sodium taurocholate. On the other hand, the addition of heat-inactivated human kidney phosphatase, Preparation MKA-Pd, to human intestinal phosphatase, Preparation MIB-d, did not lead to a retardation of the latter by sodium taurocholate. As the other results bring out more fully, retardation by taurocholic acid occurs if the active component is bone phosphatase; it fails to occur if the active component is intestinal phosphatase, the presence of other tissue extracts notwithstanding.

The possibility existed that the intestinal preparations might contain sufficient concentrations of bile acids which were already exerting optimal retardant effect and that further addition was without influence. Though this did not seem likely since the tissues were usually washed with saline before extraction and were subsequently dialyzed, several preparations of intestinal phosphatase, Preparations RbIA-d, CaIB-d, and MIB-d, were tested for bile acids by the Gregory-Pascoe reaction (34). The former two preparations were negative; Preparation MIB-d, a tissue which had not been washed, yielded a faint positive reaction such as that given by only a very small fraction of the bile acid added to the hydrolysis mixture.

There is some indication in the results of Table III that the extent of retardation is dependent on the substituent groups in the cholanic acid molecule. Thus, 0.00625 M dehydrocholic acid (3,7,12-triketocholanic acid) decreased the activity of bone or kidney phosphatase to an average of about 65 per cent of the original value, while 0.00625 M taurocholic acid, the taurine-conjugated product of 3,7,12-trihydroxycholanic acid, decreased the activity of these phosphatases to about 50 per cent, and the same concentration of glycocholic acid to about 45 per cent.

Source of Serum Phosphatase—Table V shows that the activity of serum phosphatase of rabbit, cat, and man, the last both in the normal and in two cases of Paget's disease, is decreased in the the presence of 0.00625 M sodium taurocholate to about 50 per cent

of its original value.   In addition, it was found that 0.00125 M sodium desoxycholate and 0.00625 M sodium dehydrocholate decreased the activity of the serum phosphatase of Subject T to 77 and 74 per cent, respectively.   On the basis of these results, serum phosphatase, in the species mentioned and in the two cases of Paget's disease, is to be classed with bone and kidney phosphatases, and not with intestinal phosphatases.

TABLE V

Effect of Taurocholic Acid on Activity of Serum Phosphatase

Temperature, 25°, except for Subject T, 28°; concentration of substrate, sodium β-glycerophosphate, 0.0127 M; concentration of taurocholic acid as sodium taurocholate, 0.00625 M; concentration of diluted serum, 12.5 per cent by volume of hydrolysis mixture; optimal pH, 9.0 to 9.2; and optimal concentrations of magnesium, 0.009 M, and glycine, 0.00625 M. The phosphatase activity of the serum is given on the basis of a concentration of 12.5 per cent *undiluted* serum, and the activity in the presence of sodium taurocholate is expressed in per cent of the original activity.

Serum	$Q_{0.05}$	Relative activity in presence of sodium taurocholate	Remarks
Normals			
		per cent	
Rabbit	0 00154	52	Normal animal and human sera
Cat	0.00078	50	diluted with equal parts of saline;
Human, A	0 00095	57	human A, 6 parts with 4 of saline
" B	0.00200	51	
Paget's disease			
Subject T	0.0512	45	Sera diluted with 3 parts of saline;
" M	0.0118	44	T-2 activity determined after 3
" T-2	0.0428	52	mos. in ice box

In connection with the assumption that phosphatases from the various tissues may enter the blood stream, it was of interest to determine whether the characteristic effect of the bile acids on the activity of such phosphatases would persist in the presence of serum.   Table VI shows that a concentration of 6.25 per cent serum in the hydrolysis mixture, a concentration equal to or greater than the one in which the phosphatase activity of the various sera was determined, does not affect the extent of re-

tardation of bone phosphatase by sodium taurocholate or the lack of retardation, in the presence of this bile acid, of intestinal phosphatase activity. Thus, according to Table III, the activities of the cat bone phosphatases, Preparations CaBA-d and CaBA-d3, were decreased to about 55 per cent in the presence of 0.00625 M sodium taurocholate. When cat serum was present (Table VI)

TABLE VI

Effect of Taurocholic Acid on Activity of Phosphatases in Presence of Serum

Temperature, 25°, except for Preparation MIA-d, 28°; concentration of substrate, sodium β-glycerophosphate, 0.0127 M; of sodium taurocholate, 0.00625 M; of serum, 6.25 per cent by volume of hydrolysis mixture; of phosphatase preparation, 12.5 per cent by volume of hydrolysis mixture, except for Preparation MIA-d, 37.5 per cent; optimal pH, 9.0 to 9.2; and optimal concentrations of glycine, 0.00625 M, and magnesium, 0.009 M.

Phosphatase preparation	Addition of		$Q_{0.05}$
	6.25 per cent serum	Sodium taurocholate	
Cat bone, CaBA-d3	None	None	0.0205
	Cat serum	"	0.0230
	" "	Present	0.0105
" " CaBA-d	None	None	0.0203
	Human serum	"	0.0206
	" "	Present	0.0103
Human intestinal, MIA-d	None	None	0.0175
	Human serum	Present	0.0193
" " MID-d	None	None	0.0227
	Human serum	"	0.0215
	" "	Present	0.0217
Cat " CaIB-d	None	None	0.0126
	Cat serum	Present	0.0135

the activities were decreased to about 51 per cent in the presence of this bile acid. Preparation MID-d showed an activity, $Q_{0.05}$, of 0.0227; in the presence of 6.25 per cent human serum, the activity was 0.0215, and when both human serum and 0.00625 M sodium taurocholate were present, the activity was 0.0217.

That the characteristic effect of the bile acids on the activity

of a given tissue phosphatase is not influenced by the presence of another active phosphatase is demonstrated in the results of Table VII. To illustrate, the activities of a 12.5 per cent concentration of cat bone phosphatase, Preparation CaBA-d3, were 0.0205 in the absence of, and 0.0105 in the presence of sodium taurocholate. The corresponding values for a 12.5 per cent

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Table VII

Effect of Taurocholic Acid on Activity of Mixtures of Phosphatases
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Temperature, 25°; concentration of sodium taurocholate, 0.00625 M, of sodium β-glycerophosphate, 0.0127 M; optimal pH, 9.0 to 9.2; and optimal concentrations of glycine, 0.00625 M, and of magnesium, 0.009 M. See the text for the key to the designation of phosphatase preparations. T represents a 1:4 dilution of the serum phosphatase of a patient with Paget's disease. The activities of the mixtures are calculated on the basis of the activities and the taurocholic acid activity factors for the different phosphatases as given in Tables II to VI.

Composition of mixture of phosphatases	$Q_{0.05}$	Activity in presence of sodium taurocholate			
		Calculated		Observed	
		$Q_{0.05}$	Relative	$Q_{0.05}$	Relative
			per cent		per cent
12.5% CaBA-d3 + 12.5% CaIB-d2........	0.0331	0.0232	70	0.0218	66
25% " + 12.5% "	0 0534	0.0340	63	0.0320	60
2.5% CaBA-d + 10% CaIB-d............	0.0136	0.0114	84	0.0097	71
10% " + 2.5% "	0.0186	0.0114	61	0.0105	57
10% MKA-Pd + 2.5% MIB-d.............	0.0258	0.0134	52	0.0123	48
2.5% " + 10% "	0.0101	0.0073	72	0.0071	70
12.5% MID-d + 12.5% CaBA-d3.........	0.0430	0.0333	77	0.0310	72
16% " + 21.5% "	0.0642	0.0472	73	0.0456	71
7.1% " + 17.9% T serum...........	0.0281	0.0208	74	0.0205	73
25% MIC-d7 + 12.5% " "	0.0168	0.0117	69	0.0114	68

concentration of cat intestinal phosphatase, Preparation CaIB-d2, were 0.0126 and 0.0124. The *calculated* activities of a mixture consisting of 12.5 per cent of each of these preparations were 0.0331 in the absence of, and 0.0232 in the presence of sodium taurocholate. The value *found* for the activity of the mixture when the bile acid was present was 0.0218. The other results of Table VII show, on the whole, fairly good agreement between the

values actually observed for the activities of mixtures of phosphatases in the presence of sodium taurocholate and those calculated on the basis that this bile acid exerts its characteristic effect on each of the phosphatase components of the mixture.

The above results emphasize the probability that the effect of the bile acids on the activity of phosphatase preparations (retardation of bone and kidney phosphatase activity, and absence of retardation of intestinal phosphatases) is an effect on the active enzymic component of the preparations. These results also indicate the possibility, as will be pointed out more fully later, of judging the source of the phosphatase in serum in various conditions by determining the extent of retardation of the activity of serum phosphatase in the presence of bile acids.

<div align="center">DISCUSSION</div>

Kay (12) has considered the phosphatases of the various tissues and of plasma to be quite probably identical. Evidence for the identity of the enzymes of bone, intestine, kidney, and plasma was based on (a) the almost complete removal of phosphatase from the different tissues by the same method of extraction, (b) the same optimal pH for the action of preparations from the three tissues and plasma, (c) hydrolysis of pyrophosphate at a pH optimum (7.2 to 7.8) which was the same for the three tissues and plasma, (d) stimulation of activity by magnesium ion and maximal stimulalation at the same concentration of magnesium by all four phosphatases, (e) the same ratio of rates of hydrolysis of a series of esters by each of the four phosphatases, (f) practically the same dissociation constant, for the four phosphatases, for the enzyme substrate compound, (g) synthesis of phosphoric esters by all four phosphatases.

Many of the above points have been verified incidentally in the course of work of other investigators. The data submitted in the present study, however, reveal, with respect to the retardation by the bile acids, a distinct difference between kidney, bone, and serum phosphatase, on the one hand, and intestinal phosphatase, on the other.

The question of the identity of the tissue phosphatases or, in general, of similarly acting enzymes may be considered from several view-points. In a strict sense and according to definition,

two or more given enzyme preparations may be designated as identical only if it can be shown exhaustively that they act the same way quantitatively in every instance; that is, if they show the same pattern of rates of hydrolysis on a series of substrates, if their activities are influenced to the same extent by change in substrate concentration, retardants, preventors of inactivation, by the presence of accompanying substances, or by any other alteration in the reaction environment.

The work here presented as well as that of previous investigators on other enzymes shows that it is very difficult to ascribe "identity" in this strict sense to similar preparations. Perhaps the clearest illustration of this difficulty is the finding of Falk (35) that a protein added to a given preparation of pancreatic lipase produces a different pattern of rates of hydrolysis of a series of esters. Falk, in this connection, emphasizes the necessity for considering the enzyme system as a whole. Falk and his collaborators (36) showed in earlier work that the pattern of rates of hydrolysis of a series of esters varies with the age of the animal, the species source of the tissue, and the age of the lipase extract.

From a second view-point, it may be assumed that in enzyme preparations showing identity of action in most respects, such differences as do exist are due to the presence of accompanying substances which vary with the particular extract. Used in this connection, the term "identity" may be conceived of as applying to the possession of a common chemical unit. In our present lack of knowledge concerning the relation between chemical structure of enzymes and their activities, the available experimental approach to a decision regarding the possession of a common chemical unit is to attempt the finding of an activity characteristic which persists in spite of apparent variation in accompanying substances as these appear in different tissues or in preparations of varying degrees of purity.

The term "identity of enzymes" may be thought of in still another sense. Thus there exists the possibility that the chemical structure of an enzyme may undergo partial modification, so that its actions in certain respects remain the same but differ in others. Nelson and Papadakis (37) have shown that a highly purified yeast invertase preparation, free from melibiase, is changed by partial heat inactivation into a preparation which is different from the

original in its relative actions on the two substrates, raffinose and sucrose; the form of the time course of the reaction and the degree of retardation by α-methylglucoside are, however, the same for the two preparations. Enzyme preparations of different tissues may, therefore, differ chemically in some respect and yet act identically on the substrates which are involved in physiological processes going on in these tissues.

In the present paper the question of the identity of the phosphatases is considered from the first two points of view. Variations in the pattern of hydrolysis of several phosphoric esters by a given tissue phosphatase occurred with variation in the species source and were such as to make impossible the differentiation of one tissue phosphatase from another, even within a given species. The existence of such variations was not surprising, in view of the work of Falk and his collaborators on tissue lipases. In contrast, however, it proved possible to show that the activities of bone and kidney phosphatases were considerably retarded, and those of intestinal phosphatases unaffected by bile acids, independently of the age of the extract or of the animal, of the species source of the enzyme, the presence of another extract, whether enzymically active or not, and of the presence of serum. These results, then, are probably indicative of some chemical difference between the phosphatases of bone and kidney, on the one hand, and that of intestine, on the other.

Whether such a difference is physiologically significant cannot be stated at present. If it is assumed, as seems likely, that the phosphatase in serum has an extracirculatory origin and that a given tissue phosphatase does not change its capacity for retardation (or lack of retardation) by bile acids when it passes into the blood stream (and *in vitro* it has been shown that the retardant effect is not influenced by serum), then the determination of the degree of retardation of the activity of serum phosphatase provides a possible criterion for judging its source. To illustrate, a decrease to about 50 per cent in the activity of the serum phosphatase in the normal or in Paget's disease (Table V) indicates either (a) that the phosphatase does not come from the intestinal mucosa, but from bone, kidney, or some other tissue the phosphatase of which is retarded to that degree by taurocholic acid or (b) that the phosphatase in the serum comes from several tissues, that

some of these tissue phosphatases are retarded to more than 50 per cent, others less, but that the resultant decrease in their activity by sodium taurocholate is, fortuitously, the same as that of bone or kidney phosphatase.

To define more precisely the source of serum phosphatase in the normal and in conditions which exhibit hyperphosphatasemia, it is necessary to extend the studies here reported to other tissues which may be conceived of as sources of phosphatase in the serum. It may also be possible to emphasize the difference between different phosphatases by using other concentrations of bile acids. Moreover, there is indication, from the values presented in Table III, that further study with other substituted products of cholanic acid may prove of value in determining the extent to which the similarity of bone, kidney, serum, and other tissue phosphatases persists, or else serve in their differentiation.

SUMMARY

1. The pattern of the rates of hydrolysis of sodium β-glycerophosphate, sodium hexosediphosphate, potassium benzylphosphate, and sodium propylphosphate has not been found to be constant for a phosphatase from the same tissue of different species, or to be differentiable from the pattern for a different tissue of the same individual or species.

2. The cinchona alkaloids, quinine, cinchonine, and quitenidine do not, in low concentrations, decrease the rate of hydrolysis of sodium β-glycerophosphate by tissue phosphatases. 0.00125 M mercuric chloride decreased, in several instances, the activities of tissue phosphatases, but these effects could not serve as a basis for differentiating them.

3. Taurocholic, glycocholic, desoxycholic, and dehydrocholic acids decrease considerably the action of bone and kidney phosphatases but do not affect that of intestinal phosphatases (with the exception of slight retardation of the action of a human intestinal preparation by dehydrocholic acid). This effect is dependent on the tissue, is independent of the age of the extract, the mode of preparation, and the animal species. The effect on a given tissue phosphatase is, as shown by studies with taurocholic acid, independent of the presence of other tissue extracts, whether enzymically active or not, and of the presence of serum. This effect thus serves as a means of differentiation.

4. The question of the identity of similarly acting enzymes, in general, and of that of bone, kidney, intestinal, and serum phosphatases, in particular, is discussed.

5. The probable source of the phosphatase in normal serum and, as an illustration of a pathological condition, in that of Paget's disease is considered. To define more precisely the source of serum phosphatase in various conditions, it is proposed that studies, similar to those here reported, be extended to other tissues and for other substituents of the cholanic acid molecule.

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THE CHEMISTRY OF MOLD TISSUE

XII. ISOLATION OF ARGININE, HISTIDINE, AND LYSINE FROM ASPERGILLUS SYDOWI

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For some time the nitrogen compounds of the mycelium of *Aspergillus sydowi* have been under investigation in this laboratory. The most advantageous method found for bringing the amino acids into solution was to allow the ground mycelium to autolyze in the presence of thymol for 3 or 4 days. At the end of this relatively short period the amount of nitrogen in solution as well as the amino nitrogen was at a maximum. Further prolonging the autolysis period resulted in the production of increased amounts of ammonia.

The most serious disadvantage of autolysis was the large proportion of the nitrogen that appeared as ammonia. When fresh mycelium was merely ground and allowed to autolyze at pH 7, as much as 30 per cent of the nitrogen was converted into ammonia. By drying the fresh mycelium at room temperature before carrying out the autolysis the production of ammonia could be reduced about 50 per cent; but even with this procedure considerable ammonia was produced. The source of such large amounts of ammonia was unknown, but there was some evidence that at least part of it arose from the decomposition of arginine. From autolysis solutions it was impossible to isolate arginine, though this amino acid could be readily isolated from frozen mycelium (which apparently had undergone partial autolysis) and from a water extract of perfectly fresh mycelium as well as from an acid hydrolysate of water-extracted mycelium. The speed of autolysis and

the proportion of the total nitrogen which was brought into solution during autolysis were not affected appreciably by the preliminary drying procedure.   Lysine and histidine were not destroyed during autolysis for it was possible to isolate these amino acids in considerable amounts from autolysates.

Lysine has been reported in *Aspergillus niger* (1), *Aspergillus oryzæ* (2), and in *Penicillium chrysogenum* (3); arginine and histidine have been reported in the latter two organisms (2–4).   Histidine has been isolated from *Rhizopus japonicus* (5).   However, the evidence for the presence of arginine in all cases was a Van Slyke nitrogen distribution determined on the acid hydrolysates of protein preparations obtained from the organisms; no report has been found of the actual isolation of arginine from mold.   Also, except in the case of *Aspergillus niger* (1), no report of the actual isolation of lysine from mold tissue has been discovered.   The only sure proof of the presence of an amino acid in molds is the actual isolation and characterization of the amino acid.   Because of the wide variety and unknown nature of the nitrogenous compounds in the mycelium, deductions from nitrogen distribution experiments or from color tests applied to extracts or hydrolysates, while valuable as indicators, do not conclusively prove that specified amino acids are present.

In view of the difficulty of isolating amino acids from such a complex mixture of nitrogenous compounds as exists in the autolysate of mold tissue, the quantities of lysine, arginine, and histidine isolated should be regarded as the minimum rather than as the actual amount present in the mycelium.

<div align="center">EXPERIMENTAL</div>

The mold was grown in large sterilizer incubators on glucose-inorganic salts medium as previously described (6).   After 9 days the pads were collected, washed with cold water, spread thinly in pans before electric fans to dry, and when dry, ground.   525 gm. of dry mycelium, containing 29.7 gm. of nitrogen, were suspended in 6 liters of water saturated with thymol, and the solution was adjusted to pH 7 with Na_2CO_3.   As autolysis proceeded, more Na_2CO_3 was added in order to keep the pH at approximately 7. At the beginning there were 1.53 mg. of soluble nitrogen per cc., of which 0.7 mg. was amino nitrogen (Van Slyke).   The soluble

and amino nitrogen rose rapidly in 3 to 4 days to constant values of 3.03 mg. and 1.7 mg. per cc. respectively. The ammonia nitrogen amounted to 0.49 mg. per cc.

The solution was filtered and the residue was washed with hot water. The dried residue weighed 295 gm. and hence represented 56 per cent of the original material. The filtrate contained 18.7 gm. of nitrogen or 63 per cent of the nitrogen of the mycelium. This solution was concentrated under reduced pressure to about 2 liters, made distinctly alkaline with Na_2CO_3, and concentrated further in order to remove ammonia. It was then centrifuged and washed to remove inorganic material precipitated·by the Na_2CO_3, and the resulting filtrate was treated with Na_2CO_3 and mercuric acetate (Neuberg's reagent). After standing overnight the precipitate was filtered and washed.

The precipitate, which contained the amino acids, was suspended in water and decomposed with H_2S. The HgS was filtered off and washed with hot water. The resulting filtrate contained 10.2 gm. of nitrogen of which 71 per cent was amino nitrogen (Van Slyke). The filtrate was concentrated under reduced pressure to about 2 liters, treated with an excess of hot saturated barium hydroxide solution, and allowed to stand overnight in the cold. The precipitate, which was filtered off and washed with water, contained only a trace of nitrogen. The filtrate was concentrated under reduced pressure to about 500 cc., poured into 2 liters of alcohol, and allowed to stand for 5 days in the cold. The clear supernatant liquid was then decanted; the precipitate was dissolved in water and reprecipitated by pouring into alcohol. After several days the supernatant liquid was decanted and added to the supernatant liquid from the first precipitation. The precipitate was reserved for the investigation of the dicarboxylic amino acids of the mycelium.

Isolation of Histidine—The combined filtrates were concentrated under reduced pressure to about 500 cc. in order to remove alcohol, and treated with an excess of H_2SO_4 to remove barium. To the acid solution $HgSO_4$ in dilute H_2SO_4 was added as long as a precipitate formed, and the mixture was allowed to stand overnight. The cream-colored precipitate was then filtered off and washed with dilute H_2SO_4. The filtrate was reserved for the investigation of its histidine, arginine, and lysine contents. The precipitate

was suspended in water, decomposed with H_2S, and the HgS filtered off. The filtrate was adjusted to pH 7 with barium hydroxide, $BaSO_4$ was removed, and the resulting filtrate, which contained 1.65 gm. of nitrogen, was concentrated under reduced pressure to about 200 cc., and extracted with five 500 cc. portions of cold butyl alcohol. The residual aqueous solution then no longer gave color tests for tryptophane. This solution was concentrated under reduced pressure to 40 cc., treated with a slight excess of H_2SO_4 to remove barium, mixed with a hot concentrated solution of flavianic acid, and then allowed to stand for several weeks. The yellow crystals which had formed were filtered off and washed with a dilute solution of flavianic acid. They melted at 254° with decomposition on rapid heating; histidine diflavianate melts at 254° with decomposition on rapid heating (7).

$C_{26}H_{21}O_{18}N_7S_2 \cdot \frac{1}{2}H_2O$.   Calculated, S 8.08; found, S 8.12

4.14 gm. of histidine diflavianate, corresponding to 810 mg. of histidine, were obtained. Hence at least 0.74 per cent of the nitrogen of the mycelium was histidine nitrogen.

The diflavianate was dissolved in dilute HCl, flavianic acid was extracted with butyl alcohol, and the aqueous solution was placed in a desiccator over concentrated H_2SO_4. After some time crystals formed; these were filtered off, washed with acid alcohol, and recrystallized from HCl solution by adding alcohol and ether. The crystals melted at 245°, and when mixed with known histidine dihydrochloride, the melting point was 246°. Known histidine dihydrochloride melted at 246°. Abderhalden and Einbeck (8) have reported 245°. These melting points were the temperatures at which the last crystal disappeared when the determinations were carried out in an efficiently stirred bath in which the temperature was raised at the rate of 6° per minute. Trials with known histidine dihydrochloride showed that softening occurred considerably below the melting point. Furthermore, the melting point was found to vary with the rate of heating; when the temperature was raised 1° per minute, known histidine dihydrochloride melted at 240°. The crystals gave a strong reaction for histidine when tested by the bromination-ammonia treatment method of Kapeller-Adler (9).

$C_6H_9O_2N_3\cdot2HCl$.   Calculated. NH_2-N 6.14, Cl 31.11
                          Found.   "   6.17, " 30.8

The mercury was removed from the $HgSO_4$ filtrate with H_2S, and most of the H_2SO_4 with barium hydroxide. The resulting filtrate was concentrated under reduced pressure to about 500 cc., and on it a silver salt separation of arginine and histidine was performed according to Vickery and Leavenworth (10). The histidine fraction gave no Kapeller-Adler test for histidine, and, furthermore, gave no ninhydrin reaction; however, it contained 153 mg. of nitrogen. The arginine fraction contained 102 mg. of nitrogen, but no arginine flavianate could be obtained from it and the solution gave no blue color in the ninhydrin test. The arginine had not been carried down in the $HgSO_4$ precipitate, because in previous experiments in which this precipitation was omitted no arginine could be found.

Isolation of Lysine—The filtrate from the silver salt separation was freed of barium and silver, acidified with H_2SO_4, concentrated under reduced pressure to about 600 cc., treated with phosphotungstic acid until no precipitate formed, and allowed to stand for 2 days in the cold. The precipitate was then filtered off and decomposed in the usual manner with barium hydroxide. The resulting barium- and sulfate-free filtrate, which contained 1.26 gm. of nitrogen, was concentrated under reduced pressure to about 20 cc. and treated with a hot alcoholic solution of 9 gm. of picric acid. After standing overnight the yellow crystals were filtered off and washed with alcohol; 10.56 gm. were obtained. This corresponded to 4.12 gm. of lysine, and hence at least 2.7 per cent of the nitrogen of the mycelium was lysine nitrogen. After crystallization from dilute alcohol the picrate melted at 250–251° with decomposition. Known lysine picrate melted at the same temperature. For analysis a weighed amount (about 10 mg.) of the picrate was dissolved in dilute H_2SO_4 and the picric acid was extracted with ether; nitrogen was then determined by the Kjeldahl method on the aqueous solution. The reliability of this method was first determined by trials on known lysine picrate.

$C_{12}H_{17}O_9N_5$.   Calculated, lysine N 7.47; found, lysine N 7.49

2 gm. of the picrate were suspended in dilute HCl and the picric acid was extracted with ether. The aqueous solution was concen-

trated and alcoholic H_2PtCl_6 was added. The chloroplatinate that separated was recrystallized from dilute alcohol and was found to melt at 220–221° with decomposition. Lysine chloroplatinate melts at 219–220° with decomposition (11).

$C_6H_{14}O_2N_2 \cdot H_2PtCl_6 \cdot C_2H_6OH$.   Calculated, Pt 32.39; found, Pt 32.38

Isolation of Arginine—Arginine was first isolated from some mycelium which had been harvested and stored at $-11°$ for several weeks. This material was ground, suspended in water, boiled, and filtered. The filtrate was precipitated with Neuberg's reagent, the precipitate was decomposed with H_2S, and the resulting filtrate was treated with phosphotungstic acid. From the decomposed phosphotungstic acid precipitate, arginine was isolated as the flavianate, and finally, as the picrolonate. However, repeated attempts to isolate arginine from autolysates by the same procedure failed. In order to get a better idea of the amount of arginine in the mycelium the following procedure was employed.

The mycelium was lifted from the medium, quickly washed with water, immediately ground, weighed, and dropped into boiling water. The quantity of mycelium used contained 289 gm. of dry matter, and 15.6 gm. of nitrogen. The water-extracted residue was filtered off and washed with hot water. The extract contained 3.40 gm. of nitrogen of which 1.03 gm. were amino nitrogen.

The water extract was concentrated under reduced pressure to about 500 cc., made strongly alkaline with barium hydroxide, treated with half its volume of alcohol, and allowed to stand in the cold for 18 hours. The precipitate was then centrifuged off, washed with alcohol, and discarded. Alcohol was removed by concentration under reduced pressure to about 600 cc. and most of the barium with H_2SO_4. Excess $AgNO_3$ was added, and the solution was saturated with barium hydroxide. The precipitate was centrifuged, washed, suspended in dilute H_2SO_4, decomposed with H_2S, and filtered. After part of the H_2SO_4 had been removed as $BaSO_4$ from the filtrate, it was concentrated under reduced pressure to about 300 cc., and phosphotungstic acid was added until no more precipitate formed. After some time the precipitate was filtered off and decomposed with barium hydroxide. The resulting barium-free filtrate contained 78 mg. of nitrogen. The probable presence of a small amount of histidine in this

solution was indicated by the blue color obtained with the Kapeller-Adler test for histidine (9), but in subsequent operations only arginine was isolated. In order to isolate arginine, the solution, which was free of barium and of sulfate, was concentrated under reduced pressure to about 25 cc. and heated with a solution of flavianic acid. After a week the orange crystals were filtered off, washed, and recrystallized from water; 89 mg. were obtained. The crystals melted at 258–260° with decomposition; arginine flavianate melts at 258–260° with decomposition (12).

$C_{16}H_{20}O_{10}N_6S$. Calculated, S 6.56; found, S 6.49

The water-extracted mycelium was suspended in 700 cc. of 5 N H_2SO_4 and heated in an autoclave at 20 pounds pressure for 8 hours. The hydrolysis mixture was diluted with water, partly freed of H_2SO_4 with barium hydroxide, centrifuged, and washed. The solution contained 7.45 gm. of nitrogen. Most of the H_2SO_4 was removed and the resulting filtrate was concentrated under reduced pressure to about 350 cc. and treated with phosphotungstic acid until no more precipitate formed. After the mixture had stood for some time in the cold, the precipitate was filtered off and decomposed with barium hydroxide. The resulting filtrate was freed of barium, concentrated under reduced pressure to about 300 cc., heated to boiling, and treated with a hot solution of 10 gm. of flavianic acid. After 2 weeks the orange crystals were filtered off and washed with water; 4.46 gm. were obtained. The crystals melted at 255–260° with decomposition. When analyzed by the method of Langley and Albrecht (13), they were found to contain 64.5 per cent flavianic acid. Arginine flavianate contains 64.3 per cent flavianic acid.

$C_{16}H_{20}O_{10}N_6S$. Calculated, S 6.56; found, S 6.64

The crystals were treated with an excess of warm barium hydroxide solution, and the barium flavianate was filtered off on a layer of norit. Excess barium was removed from the filtrate with CO_2, and the solution was concentrated under reduced pressure to about 5 cc. An alcoholic solution of picrolonic acid was added; the solution was boiled and allowed to cool. Lemon-yellow needles were obtained which, after recrystallization from water,

melted at 238° with decomposition. Arginine picrolonate melts at 237.5° with decomposition (14).

The total amount of arginine flavianate obtained from the water extract and the residual mycelium was 4.55 gm., which was equivalent to 1.63 gm. of arginine. Hence at least 1.8 per cent of the nitrogen of the mycelium was arginine nitrogen.

SUMMARY

Arginine, histidine, and lysine have been isolated from *Aspergillus sydowi* and identified by examination and analysis of suitable derivatives. Histidine and lysine were isolated from an autolysate of the mycelium, but arginine could not be obtained from such a solution, as it was destroyed during autolysis. Arginine was isolated from both the water extract of the mycelium and the acid hydrolysate of the water-insoluble residue. Most of the arginine was present in the combined form.

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METABOLISM AND MODE OF ACTION OF
VITAMIN D

II. STORAGE OF VITAMIN D IN DIFFERENT TISSUES IN VIVO

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In order to learn more about the fate of vitamin D within the
body tissues, and thus possibly to throw some light on its still
obscure mode of action, studies were undertaken and have been
reported (1) showing that when viosterol in oil was given by mouth
to rabbits in a single dose of 20 cc.[1] detectable amounts of vitamin
D were found to be circulating in the blood for from 2 to 3 months.

The object of the continuation experiments, the results of which
are here reported, was to detect the duration of storage of vitamin
D in different tissues under the same conditions that prevailed in
the previous investigations.

EXPERIMENTAL

To twelve male rabbits, weighing from 3.5 to 5 kilos, 20 cc. of
viosterol in oil were administered by stomach tube. The rabbits
were killed at the end of 1, 3, 5, 6, 7, 8, 9, and 12 weeks by cutting
the femoral vessels after the animals had been anesthetized with a
small amount of ether. The animals were immediately placed in
the refrigerator, and the tissues were removed not later than 10
hours after death.

The presence of vitamin D was determined in extracts of oxa-
lated plasma, unwashed erythrocytes, brain, small and large
intestines (free of chyme and feces), lungs, skin from abdominal
region only (without hair and subcutaneous fat), kidney, and liver.

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[1] The 20 cc. dose represents 200,000 U.S.P. units (United States Pharma-
copœia X, revised (1934)) or international units (corresponding to approxi-
mately 1.6 mg. of irradiated ergosterol).

Method

The tissues were weighed fresh, ground through a food chopper or cut into small pieces with scissors, and treated for about 2 hours on the steam bath with 20 per cent potassium hydroxide in alcohol. In order to prevent coagulation while the solutions were cooling, ether was added carefully, with constant stirring, to the still hot but liquid material. 5-Fold ether extraction was then performed in Mojonnier flasks, with 300 to 600 cc. of ether. The ether extracts were washed with water, in separatory funnels, until the water remained colorless, and were slowly evaporated on the water bath. The residue from ether evaporation was then dissolved in Wesson oil in an amount equal to one-sixth of the original weight of the tissues. The concentration of the oil suspensions was thus kept comparable and constant for each single tissue.[2]

By pipette 0.1 cc. of the tissue extracts was fed daily for 10 days to rats which had been kept for 3 weeks on a rickets-producing diet (Steenbock Ration 2965). Roentgenograms of the lower extremities were made on the day the administration of the extracts was begun and on the 8th and 10th days following. After the last roentgenograms had been taken, the blood obtained from the rats receiving the same tissue extract was pooled, and serum phosphorus and calcium determinations were made by the titrimetric method of Samson (2).

Results

The results of these tests are recorded in Table I. The storage time of vitamin D in the tissues is shown in the last column. It must be realized that an excessive amount of vitamin D in the form of viosterol had been given. In all the tissues examined, the vitamin D was deposited and found present for at least 1 week.

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[2] The method was tested by adding known amounts of viosterol in oil to tissues (liver and muscle) from animals not used in the experiments. It was found possible in this way to recover vitamin D almost unit per unit. The efficacy of the method is evident also when the results given in this paper are compared with those previously reported (1). In the earlier experiments, blood serum from which vitamin D had not been extracted was injected in rachitic rats, with results that agree with those now obtained by the extraction method.

The order in which the vitamin D depots were depleted *in vivo* was as follows: brain, erythrocytes, small intestines, large intestines, skin, lungs, kidney, liver, blood plasma.

It is rather surprising to find that this fat-soluble vitamin disappeared first in the brain, which, on account of its chemical composition, would presumably be the most likely place of retention. This observation shows that other conditions than the purely chemical properties of the tissues are decisive in influencing the retention of vitamin D. This fact is also evident from the results obtained in all the other tissues; the length of time that vitamin D was stored by no means paralleled the fat or lipid content of the tissues.

The erythrocytes were apparently depleted completely of vitamin D after 6 weeks, whereas vitamin D was present in the blood plasma for as much as and possibly more than 3 months. In this connection it may be of interest to note that Hess (3) found that in · the blood of cows fed irradiated yeast the plasma contained 4 times as much vitamin D per gm. as did the erythrocytes.

The time required for depletion of vitamin D in the skin, lungs, kidneys, and small and large intestines did not show much variation. It can only be stated that vitamin D was stored in all these tissues to about the same extent; that is, for about 5 to 8 weeks.

It seems as if the liver can hold vitamin D more tenaciously than can the other organs. The results obtained, however, are not sufficiently uniform and the difference in storage time in the liver, on the one hand, and in the skin, lungs, kidneys, and small and large intestines, on the other, is not sufficiently great to justify a more positive statement at this time. To have found that only the liver retained vitamin D for from 6 to 8 to 12 weeks is certainly suggestive of the hypothesis advanced by Gerstenberger (4) that the liver probably plays a decisive rôle in the functioning of vitamin D.

Coppens and Metz (5) reported that lungs and blood, when incubated *in vitro*, decomposed vitamin D. Their results do not agree with the findings presented here, obtained from experiments *in vivo*, nor is their assumption of the presence of an enzyme in lungs and blood which might lead to inactivation of vitamin D substantiated. On the contrary, it has now been established that

blood plasma, *in vivo*, contains vitamin D in active form longer than does any other tissue.

Considering the rather impressive length of time that vitamin D is stored in the body tissues, it can be assumed that if there is consumption of vitamin D within the tissues at all, it must be very slight. Investigations now in progress indicate that excretion is

TABLE I—*Antirachitic Potency for Rats of Different Tissue Extracts Obtained from R Tube—10 Days after Treatment*

0 no healing; + slight; ++ moderate; +++ almost complete.

Tissue extract	Healing in rats fed tissue extract from rabbits not given viosterol (controls)			Healing in rats fed tissue ext							
	x-Ray healing	P	Ca	1 wk.			3 wks.			5 wks.	
				x-Ray healing	P	Ca	x-Ray healing	P	Ca	x-Ray healing	P
		mg. per cent	*mg. per cent*		*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*		*mg. per cent*
Brain	0 0			++			0 0			0	
Red blood cells	0 0	5 3	10 4				++ ++ ++	7 4	12 6	+ ++ ++	6.3
Small intestines							+ +			++ ++	6.8
Large intestines							+++ ++	5 9	11 4	+ + ++	5 4
Skin	0 0			++ ++			++ ++ ++	5 7	12 6	+++ ++ ++	6.9
Lungs Kidneys	0			+++ +++			+			++ ++	6.0
Liver	0 0	4 4	10 3	++ +++ +++	5 6	13 0	+++ +++	7 0	12 8	++ ++ ++	6.2
Blood plasma	0 0	5 3	10 4	+++ +++ +++	5 9	12 8	+++ +++ ++	6 9	12 0	+++ ++ +++	8.1
No. of rabbits used	1			1			1			1	

perhaps the only, certainly the chief means of depleting the body of vitamin D. That blood plasma contained vitamin D longer than did any other tissue is consistent with this conception. It might also be mentioned here that the protracted storage time of

vitamin D explains Harnapp's (6) recently reported cure of rickets in human beings with a large, single dose of vitamin D₂.

SUMMARY

To twelve male rabbits was administered by stomach tube a single dose of 20 cc. of viosterol in oil (200,000 U.S.P. units of

abbits Which Had Received 1 to 12 Weeks before Death 20 Cc. of Viosterol in Oil by Stomach with Tissue Extracts Had Been Started

tract from rabbits given viosterol 1 to 12 weeks before death

	6 wks.			7 wks.			8 wks.			9 wks.			12 wks.			Vitamin D stored in tissues
Ca	x-Ray healing	P	Ca	x-Ray healing	P	Ca	x-Ray healing	P	Ca	x-Ray healing	P	Ca	x-Ray healing	P	Ca	wks.
mg. per cent		mg. per cent	mg. per cent		mg. per cent	mg. per cent		mg. per cent	mg. per cent		mg. per cent	mg. per cent		mg. per cent	mg. per cent	
	0			0			0 0 0			0 0 0			0 0 0			1-2
11 6	+ +			0 0 0	4 1	13 6	± ±	6 9	12 4	0 0			0 0	2 8	12 6	5-6
	0 0			0 0	5 1	12 4				0 0	5 1	11 2				
12.0	+ +	4 9	11 4	0 0 0	4 5	13 0	+ +	6 0	13 2	0 0			0 0 0	3 9	11 6	5-8
	0 0	3 4	9.5	0 0 0	4 7	11 4				0 0 0	5 1	11 0				
13 2	+ +			0 0 0			++ ++	4 6	12 8	0 0			0 0 0	5 4	11 6	6-8
	+ 0	3 8	12.1		5 5	13.8				0 0 0	5 8	12 0				
11.8	++ ++	4.7	13 2	0 0	5 6	12 4	0 0 +			0 0 0	2 6	12.8	0 0			6-8
	++ ++									0 0			0 0			
	0 0 0				5 5	13 8										
11.4	+ +			0 0			0 0 +			+ 0	5 5	12.8	0			6-9
	++ +++	5.0	12 3	0 0 0	4 6	11 6	++	6 4		0 0	3 7	11 4	0 0			6-9
	+ 0 0									+ 0						
10.8	++ +++ ++	7 0	12 5	0 0 0	5.0	11 0	0 0 0	2 77	9.0	0 0	3.7	11 4	+ +	4 9	9.2	6-8-12
	0 + +	3.8	12.1	+ ± 0	5 5	11 0	+ + 0	5 4	13 6	0 0 0	6 8	12 2	0 0	3 4	11 2	
													0 0	5 2	12.8	
13 0	+++ +++	5 1	12.3	+ +++ +	6 7		+ + ±	3.7	10.3	0 0			+ +	4.1	11 6	8-12
	0 0	3.4	9 5	+ ++			+ + +	5.7	14 4	+ ++ +	6.0	13 8	0	4 2	10 6	and
													+ ++ ++	6 7	13.6	more
	2			2			2			2			2			

vitamin D, corresponding to approximately 1.6 mg. of irradiated ergosterol).

In order to detect the length of time that vitamin D is stored in the tissues, the animals were killed at the end of 1, 3, 5, 6, 7, 8,

9, and 12 weeks, and the tissues were removed.   Extracts of the tissues were fed to rachitic rats.   Under these conditions it was found that vitamin D was stored in the brain for 1 to 2 weeks, in erythrocytes for 5 to 6 weeks, in the small intestines for 5 to 8 weeks, in the large intestines for 6 to 8 weeks, in the skin for 6 to 8 weeks, in the lungs for 6 to 9 weeks, in the kidneys for 6 to 9 weeks, in the liver for 6 to 8 to 12 weeks, and in blood plasma for 8 to 12 weeks and more.   It is assumed that consumption of vitamin D within the tissues, if it exists at all, must be very slight.   It seems more likely that excretion is the chief means by which the body is depleted of its vitamin D depots.

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THE DETERMINATION OF CHOLESTEROL*

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The procedure of Schoenheimer and Sperry (1) yields much
less variable and lower average values for the percentage of free
in total cholesterol in the blood serum of healthy persons (2)
than have been reported by other investigators using other
methods. The differences are too great to be accounted for by
chance variation; either the other procedures or the one used in
this laboratory must give erroneous results.

In testing the accuracy of their method Schoenheimer and
Sperry compared it with the Windaus macrogravimetric pro-
cedure. The concentration of total cholesterol was determined
in several samples of serum by one of the authors (R. S.) with
the macromethod and independently by the present author with
the new micromethod. Excellent agreement was obtained. As
the microprocedure for determination of free cholesterol is the
same as that employed for total cholesterol, except that it does
not include the extra steps of hydrolysis and acidification, no
such comparative estimations of free cholesterol were thought
necessary. There is some indication, however, that the differ-
ence between the results obtained with the new method and
those with other procedures is associated more with errors in
the determination of free than of total cholesterol (2) and it be-
comes necessary, therefore, to compare the micromethod for
free cholesterol with the macrogravimetric procedure. This has
been done in the present investigation. The concentration of
free and also of total cholesterol was determined by the method

of Schoenheimer and Sperry and by one or more modifications of the macrogravimetric procedure in a number of samples of blood serum.

The microprocedure was applied in its original form except for three minor changes which have been in use in this laboratory for some time.

1. In preparing the digitonin solution centrifuging is omitted. The precipitate which forms on standing in the ice box is removed by filtration alone.

2. In making the extracts the serum is run into cold alcohol-acetone without shaking while the pipette is draining. The contents of the flask are mixed with a swirling motion as soon as the pipette is withdrawn and then brought to a boil. A very finely divided precipitate of protein is obtained and it is not necessary to insert the stopper and shake vigorously to break up clumps of protein which frequently formed with the original procedure. Also there is no coagulation of protein on the tip of the pipette as was always the case when the flask was shaken during the addition of serum.

3. Instead of adding 1 cc. of alcohol-ether with a pipette after incubation of the total cholesterol samples, the 15 cc. centrifuge tubes are now calibrated at 2 cc. and the solvent is added to the mark with a dropper. This technique is not only slightly faster but it compensates for any loss of solvent during incubation.

Extraction of Serum for Macrogravimetric Analysis—Two procedures were used. Procedure E_1 was based on the method of Fex (3) for extraction of tissues. A sample of serum, varying from 30 to 50 cc., was pipetted into an Erlenmeyer flask and from 1.5 to 2 volumes of 3 per cent NaOH solution were added. After standing at room temperature overnight the solution was heated on the steam bath for 1 to 4 hours. (In some instances heating was omitted. No effect on the result was noted; duplicate determinations with and without heating agreed closely.) After cooling it was washed into a separatory funnel with water, a little alcohol, and ether in the order given. (In this and all other transfers from one vessel to another the greatest care was taken to avoid mechanical loss. At least five portions of solvent were

used in washing and the outside of the flask or separatory funnel neck, or beaker lip, was rinsed off after each washing was poured out.) Enough ether was added to equal about one-half the volume of aqueous solution and the contents were rotated gently without vigorous shaking. The lower layer was drawn off into another separatory funnel and the extraction was repeated in the same manner three times more. Despite the avoidance of excessive agitation during the extraction, a small amount of emulsion always formed. In the analysis of the first four samples various expedients, such as drawing the emulsion off, taking it to dryness in a beaker, and reextracting, were tried. In the remaining determinations the following procedure was used: The emulsion was left with the first ether extract, only the clear lower layer being drawn off. Very little emulsion then formed at the second extraction and none at the third and fourth. The combined first and second extracts with the emulsion were filtered into the combined third and fourth extracts. The emulsion broke in passing through the filter, which was washed thoroughly, and no further difficulty was experienced.

The combined ether extracts were washed with water until neutral (four washings usually sufficed) and then taken to dryness in a 50 cc. volumetric flask with gentle heating and a slow current of air (a glass tube connected to a suction pump was inserted in the neck).

In the second extraction procedure (E_2), which was based on that of Bloor (4), a sample of serum was run slowly into about 20 volumes of cold alcohol-ether (3:1), which was swirled during the addition. The mixture was brought to a boil on the steam bath with constant agitation and then filtered through a cloth bag suspended in a small percolator. The precipitate was allowed to drain, usually overnight, and then kneaded thoroughly to prevent caking during the subsequent continuous extraction with hot alcohol (5), to which it was subjected for a period of about 8 hours. After the alcohol extraction the bag and its contents were washed through several times with ether. The combined filtrate and extracts were taken to dryness on the steam bath with the aid of an air current from a suction line. The dark colored residue was extracted thoroughly with many small portions of ether, which were filtered into a 50 cc. volumetric flask.

A considerable amount of dark colored material remained undis-solved.   The ether was removed as in Procedure E_1.

From this point on extracts obtained by Procedures E_1 and E_2 were handled in the same way.   About 35 cc. of absolute alcohol were added and the flasks were heated just to boiling on the steam bath for about an hour.   It was necessary to heat Procedure E_1 extracts so long to dissolve the colorless or slightly yellow, oily residue (probably cholesterol esters).   In the case of Procedure E_2 extracts no such oil could be seen because of the presence of a large amount of dark colored material which did not dissolve, but heating was continued as with Procedure E_1 extracts to insure complete solution of cholesterol esters.   After cooling, adjustment to the mark, and mixing the extracts were filtered and aliquots were pipetted at once for analysis.

Determination of Free Cholesterol—An aliquot of 15 or 25 cc. of the absolute alcohol extract in a 100 cc. beaker was brought to a boil on the steam bath and an amount of digitonin solution (1 per cent in 80 per cent alcohol) was added, sufficient to give an excess of at least 50 per cent over the required amount as calculated from the microanalysis.   From this point on two differ-ent procedures were employed.

In the first (Procedure F_1) sufficient water was added to make the final concentration 20 per cent; the solution was stirred thor-oughly and kept just at the boiling point for about a minute. The beaker was then placed under a bell jar and allowed to stand overnight.   The mixture was filtered through a Jena glass filter crucible (No. 1-G-3) which had been washed with 80 per cent alcohol followed by ether, dried for an hour at about 100°, placed overnight in a desiccator over calcium chloride, and weighed. Filtration was allowed to proceed by adjusting the suction at a rate no faster than would permit counting the drops.   The pre-cipitate was transferred quantitatively to the crucible with five or more small portions of 80 per cent alcohol and washed several times with the same solvent followed by ether.   It was not per-mitted to dry down on the filter until the final ether washing. The crucible was dried as described above.   It was weighed as rapidly as possible, with a damped balance, as cholesterol digi-tonide is hygroscopic.

The second procedure (F_2) was based on that described by

Gardner and Gainsborough (6). No water was added as in the method of the English workers, since the amount in the digitonin solution was sufficient to throw out a precipitate on cooling. After standing overnight the solvent was removed on the steam bath with the aid of an air current. The residue was washed thoroughly with many portions of ether which were filtered through a glass filter crucible. Combined cholesterol was determined in the filtrate (see below). A new receiver was installed and the precipitate was washed quantitatively into the crucible with many small portions of boiling water. Washing was continued until the filtrate showed no tendency to foam. The crucible was dried and weighed as in Procedure F_1.

Determination of Total Cholesterol—Three different procedures were employed. In the first (Procedure T_1) an aliquot of the absolute alcohol solution (usually 10 cc.) in a 125 cc. Erlenmeyer flask was treated with 10 cc. (5 cc. in the first two determinations) of 10 per cent alcoholic KOH, refluxed (cold finger) on the steam bath for about 2 hours, and transferred to a separatory funnel, the flask being washed with several small portions of alcohol, followed by ether. Water (40 cc. except in Procedure T_{1a} where 80 cc. were used—see below) was added and the aqueous layer was extracted four times with ether. The combined extracts were washed with water until neutral and taken to dryness in a 100 cc. beaker. The residue was dissolved in 10 cc. of absolute alcohol, precipitated, filtered, and weighed as in Procedure F_1.

In the second procedure (T_2) the total cholesterol was measured as the sum of the free cholesterol, determined by Procedure F_2, and combined cholesterol which was determined in the ether filtrate from the free cholesterol precipitation by a procedure analogous to Procedure T_1 except for a longer period of refluxing with alkali (about 8 hours).

The third procedure (T_3) was based on that employed in the micromethod of Schoenheimer and Sperry. An aliquot (usually 10 cc.) of the absolute alcohol extract was diluted with an equal volume of acetone and 1 drop of KOH solution (10 gm. of KOH to 20 cc. of water) per cc. was added. The alkali was dissolved by shaking and gentle heating and the flask (50 cc.) containing the solution was immersed in sand in a preserving jar and incubated as in the micromethod. In one instance (Sample 6) the alkaline

solution was extracted and carried through according to Procedure T_1. In another (Sample 7) the solution was made acid with 5 per cent HCl and precipitated directly with an aqueous solution of digitonin (6 mg. per cc.), as in the micromethod. The precipitate was gelatinous and difficult to handle, so in the remaining experiments sufficient water was added to make the concentration 20 per cent and the cholesterol was precipitated with the digitonin solution (1 per cent in 80 per cent alcohol) used throughout this work.

<div align="center">DISCUSSION</div>

Comparison of Macroprocedures—Analyses were carried out on three samples of pooled human serum and plasma and eleven samples of dog serum. In most instances several determinations were made on the same sample with various combinations of the procedures described. In all, thirty-seven analyses of free cholesterol and 61 of total cholesterol were carried out. In one sample of serum (Sample 10) large errors of unknown origin occurred in the determination of free cholesterol. These analyses (three were carried out) are omitted from Tables I and II.

In addition, unsuccessful attempts were made to determine free cholesterol in aliquots from four samples by a combination of Procedures E_2 and F_2. It proved impossible to carry through an analysis with this technique because the ether washing of the dried digitonin precipitate always came through cloudy. This was true, even though an exceedingly slow filtration rate was employed; the filtrate could not be clarified completely by refiltration.

There was a large variation in the cholesterol content of the samples of serum, in a few instances a considerable difference between the aliquots taken for analysis, and consequently a large variation in the quantities of cholesterol digitonide weighed. In many cases the amounts were quite small (minimum 18.4 mg.). It would be misleading, therefore, to state the variations between the findings with different procedures in the customary manner as percentage deviation, since it is probable that the inherent error of the macroprocedure is an absolute quantity and is independent, within wide limits, of the amount of cholesterol determined. Hence the deviations are presented as the differences

between the actual weights of digitonide. Where two determinations were carried out by the same combination of procedures on the same sample, the duplicate values were averaged and the difference between averages was taken. (Such duplicate determinations showed satisfactory agreement for both free and total cholesterol.) The difference was arbitrarily called + when a larger value was obtained by Procedures F_1, T_1, or E_1 than by the corresponding 2 or 3 procedure. In the cases in which unequal aliquots were compared the difference was estimated by the

TABLE I

Comparison of Different Macroprocedures

Procedures compared	No. of comparisons	Maximum difference	Average difference	t^*	Value of t necessary to establish significance†
		mg.	*mg.*		
F_1 and F_2 (on same extract).....	6	-2.6	-1.35	3.035	4.032
T_1 " T_2 " " "	6	$+4.1$	$+0.70$	0.787	4.032
" " T_3‡ " " "	18	-3.9	-0.42	1.012	2.898
E_1 " E_2 (free cholesterol, F_1).	7	-4.7	$+0.83$	0.596	3.707
" " " (total cholesterol, T_1 or T_3).............	13	-3.7	-0.95	1.780	3.055

* Calculated according to Fisher ((7), p. 118).

† A probability of 0.01 (1 chance in 100 that random sampling is responsible for the difference) was selected as the criterion of significance. The values of t in the last column (from Fisher, (7) p. 158) are those necessary to establish this probability for the different numbers of comparisons.

‡ Procedures T_2 and T_3 were compared in only one instance. The difference was 2.9 mg.

calculation $\frac{1}{2}(na - mb)(1/n + 1/m)$ in which m and n are the volumes taken in the 1 and 2 (or 3) procedures respectively, and a and b are the weights of digitonide obtained respectively from volumes m and n.

The findings are summarized in Table I. They show that in no case did the two procedures compared differ significantly from each other. Some of the individual differences were quite large (maximum 4.7 mg.) but in the majority of comparisons the values agreed closely. In only seven of 51 was the difference greater than 3 mg. while in nineteen it was 1.0 mg. or less. The

difference which came closest to being significant was obtained in the comparison of Procedures F_1 and F_2. In five of six instances the value obtained with Procedure F_2 was higher than that by Procedure F_1. The differences were quite small, however (all but one less than 2 mg.).

The greatest variation occurred in the comparison of extraction Procedures E_1 and E_2 with the same free cholesterol procedure (F_1). In four of seven cases the difference was more than 3 mg. No explanation for this result is evident. It must represent errors in the determination of free cholesterol rather than in the extraction procedures, since total cholesterol determinations on the two extracts agreed quite well in most instances. (In only one of thirteen comparisons was the difference more than 3 mg.) It should be reemphasized at this point that the greatest care was taken throughout to avoid mechanical loss. Duplicate determinations carried out with the same combination of procedures (E_1F_1, E_2F_1, E_1T_1, E_2T_1, E_1T_3, and E_2T_3) agreed closely. In eight instances the average, minimum, and maximum differences were 1.03, 0, and 1.7 mg. respectively for free cholesterol, and in thirteen instances 0.92, 0.4, and 2.3 mg. for total cholesterol (including four duplicate determinations on heated and unheated extracts by Procedure E_1, giving an average difference of 1.0 mg.).

Schoenheimer and Dam (8) found that in precipitating cholesterol from 96 per cent alcohol the amount of precipitate varies considerably with the excess amount of digitonin added. This observation has been quoted extensively and correction factors have been proposed (9). In Dr. Schoenheimer's experience (personal communication[1]) within wide limits the amount of digitonin used does not affect the result provided there is an adequate excess and provided the precipitation is carried out in solutions containing sufficient water (20 per cent, as in the present investigation, or more). Differences in the amount of digitonin used cannot account for the errors under discussion, since in several instances where such errors occurred the same amount of digitonin was used to precipitate equal aliquots.

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[1] Procedures E_1, F_1, and T_1 are essentially those which Dr. Schoenheimer has used for some time in the determination of cholesterol. The author is indebted to Dr. Schoenheimer for acquainting him with the details of these procedures.

The smallest average difference was obtained in the comparison of Procedures T_1 and T_3. In nine of seventeen cases the difference was less than 1 mg. and only once was it greater than 3 mg. This result is of particular interest, since it shows conclusively that the mild saponification procedure employed in the micromethod and also used in Procedure T_3 is adequate. Indeed in most instances up to Sample 12 Procedure T_3 gave higher results than T_1. The differences were quite small except in Sample 11, where they reached 3.9 mg. It seemed that small losses were occurring in Procedure T_1. Hence in all four of the analyses made by Procedure T_1 in Sample 12 and in two in Sample 13 the amount of water added in the extraction of cholesterol after saponification was doubled (Procedure T_{1a}). Instead of a crystal-clear separation as with Procedure T_1 the lower layer remained slightly opalescent and the values were considerably lower than those obtained with Procedure T_3 (maximum difference 17.4 mg.). Evidently the use of too much water does not improve and may definitely interfere with the extraction even though no apparent emulsion forms. Results obtained with Procedure T_{1a} are not included in any of the tabulations.

A more probable cause of the lower results with Procedure T_1 was recognized. In many instances, especially in Sample 11, the combined ether extracts occupied a large part of the space in the separatory funnel. The volume of wash water was necessarily small and the concentration of alcohol in the first washing must have been large—probably large enough to dissolve appreciable amounts of cholesterol. In Samples 12 to 14 the ether extract was divided into two portions, each of which was washed separately with a large volume of water. In Sample 12 the use of Procedure T_{1a} obscured any effect of this technique but in Samples 13 and 14 values by Procedure T_1 showed no tendency to be lower than those by Procedure T_3 and agreed very well with those obtained with the microprocedure.

Comparison of Micro- with Macroprocedures—The weights of digitonide corresponding to the macro aliquots were calculated from the microdeterminations and the differences between them and the actual weights were subjected to statistical analysis. Since no significant differences among the macroprocedures could be established, they were not treated separately; all of the

individual differences between micro and macro values were averaged together for free and for total cholesterol (except the free cholesterol values from Sample 10 (see above) and total cholesterol values obtained with Procedure T_{1a}). When the micro value was higher than the macro value the difference was called $+$.

In thirty-four comparisons the average difference for free cholesterol was -0.21 mg. with a probable error[2] of 0.33 mg. Evidently the micro- and macroprocedures did not differ significantly. The variability was not much greater than was observed among the macroprocedures. In a few instances quite large differences were observed (maximum -6.8 mg.) but in twenty-six of the thirty-four cases the difference was 3.0 mg. or less. The sum of the errors of the macro- and microprocedures may be expected to be at least this large.

In 55 comparisons the average difference for total cholesterol was $+1.88$ mg. with a probable error of 0.27 mg. The difference is statistically significant and indicates that the microprocedure gives higher results than the macroprocedure. Large differences (all positive—maximum $+13.1$ mg.) were obtained in four determinations in the early part of the work, while difficulty was being experienced with emulsions and before the final technique had been adopted. If these be omitted, the average becomes $+1.28$ mg. with a probable error of 0.15 mg. The still significant difference represents in large part the tendency of Procedure T_1 to give low values, probably because of the use of too little water in the first washing (see above). A comparison of the values obtained by the microprocedure with those by Procedure T_3 alone shows no significant difference. The results as a whole do not permit the conclusion that the microprocedure gives appreciably different results from the macroprocedure, since in forty-four of 51 comparisons (including the questionable analyses made by Procedure T_1 but not the four determinations mentioned above) the difference was 3.0 mg. or less.

Percentage of Free Cholesterol in Total Cholesterol—Although in most instances the micro- and macrodeterminations agreed quite closely, the individual differences were rather large in some of

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[2] Calculated from the formulas: $\sigma = \sqrt{\Sigma d^2/n}$; probable error $= 0.6745$ $\sigma/\sqrt{n}$.

TABLE II

Percentage of Free Cholesterol in Total Cholesterol Determined by Micro- and Macroprocedures

Sample No.	Procedure	Cholesterol Total	Cholesterol Free	Free/Total	Sample No.	Procedure	Cholesterol Total	Cholesterol Free	Free/Total
		mg. per 100 cc.	mg. per 100 cc.	per cent			mg. per 100 cc.	mg. per 100 cc.	per cent
1	Micro	187.5	53 9	28.7	11	Micro	126.0	33.7	26.7
	$E_1T_1F_1$	164.3	50.0	30.4		$E_1T_3F_1$	111.9	29.6	26.5
	$E_1T_2F_2$	164.3	52.8	32.1		$E_1T_1F_1$	109.5	28.8	26.3
2	Micro	199.1	55.5	27.9		$E_1T_3F_1$	116.1	28.8	24.8
	$E_1T_1F_1$	195.9	58.5	29.9		$E_2T_1F_1$	116.1	34.2	29.5
	$E_1T_2F_2$	192.1	60.9	31.7		$E_2T_3F_1$	121.9	34.2	28.1
3*	Micro	168.4	61.5	36.5		$E_2T_1F_1$	114.6	34.2	29.8
	$E_1T_1F_1$	162.3	63.5	39.1		$E_2T_3F_1$	124.1	34.2	27.5
	$E_1T_2F_2$	167.4	65.9	39.3	12	Micro	195.2	57.2	29.3
4	Micro	213.3	58.1	27.2		$E_1T_3F_1$	195.1	60.2	30.9
"	$E_1T_1F_1$	200.0	57.9	29.0		$E_1T_3F_1$	196.4	58.6	29.8
	$E_1T_2F_2$	192.0	61.0	31.8		$E_2T_3F_1$	201.2	58.0	28.8
5	Micro	216.9	59.3	27.3		$E_2T_3F_1$	199.5	58.8	29.5
	$E_2T_1F_1$	218.1	53.2	24.4	13	Micro	203.1	57.0	28.1
6	Micro	197.4	51.3	26.0		$E_1T_3F_1$	202.4	63.1	31.2
	$E_2T_1F_1$	193.3	49.7	25.7		$E_1T_1F_1$	207.1	63.6	30.7
	$E_2T_3F_1$	190.0	49.7	26.2		$E_1T_3F_1$	205.6	63.6	30.9
7	Micro	203.9	59.1	29.0		$E_2T_1F_1$	209.2	58.9	28.2
	$E_1T_1F_1$	197.7	63.5	32.1		$E_2T_1F_1$	199.8	59.8	29.9
	$E_1T_2F_2$	196.5	61.9	31.5		$E_2T_3F_1$	206.8	59.8	28.9
	$E_2T_1F_1$	194.8	52.9	27.2	14	Micro	204.6	60.9	29.8
	$E_2T_4F_1$	195.5	52.9	27.1		$E_1T_1F_1$	205.6	60.8	29.6
8*	Micro	112.1	40.2	35.9		$E_1T_3F_1$	202.4	60.8	30.0
	$E_1T_1F_1$	102.3	31.1	30.4		$E_1T_1F_1$	200.0	59.0	29.5
	$E_1T_3F_1$	105.0	31.1	29.6		$E_1T_3F_1$	201.2	59.0	29.3
	$E_2T_1F_1$	114.1	37.3	32.7		$E_2T_1F_1$	204.6	56.0	27.4
	$E_2T_3F_1$	114.4	37.3	32.6		$E_2T_3F_1$	200.7	56.0	27.9
9	Micro	209.4	55.4	26.5		$E_2T_1F_1$	206.4	57.8	28.0
	$E_1T_1F_1$	205.5	56.0	27.3		$E_2T_3F_1$	199.4	57.8	29.0
	$E_1T_2F_2$	204.9	62.0	30.3					
	$E_1T_3F_1$	196.4	56.0	28.5					
	$E_2T_1F_1$	202.0	53.4	26.4					
	$E_2T_3F_1$	208.8	53.4	25.6					

* These samples, showing a percentage above the normal human range, were taken from the same dog.

the comparisons. The question arises whether the variations were great enough to account for the marked discrepancy between the results of the author (2), who found that the procedure of Schoenheimer and Sperry yields relatively constant values for the percentage of free in total cholesterol in the blood serum of healthy persons, and those of other investigators, who have reported much more variable and higher average values. The data (Table II) show that this is not the case. In no instance (except in Sample 8, Procedure $E_1T_3F_1$) was the difference between the percentage values obtained with the micro- and macroprocedures greater than the difference between the minimum (24.3) and maximum (30.1) percentages observed in healthy persons (2). In most the variation was much less. It is concluded, therefore, that the wide discrepancy between the findings with the method of Schoenheimer and Sperry and with other methods cannot be accounted for by errors in the former.

SUMMARY

The concentration of free and total cholesterol was determined in fourteen samples of blood serum by the micromethod of Schoenheimer and Sperry and by one or more modifications of the macrogravimetric procedure. With the exception of one combination of procedures for free cholesterol determination, with which no results could be obtained, no statistically significant differences among the various macroprocedures could be demonstrated.

Although fairly large differences between the micro- and macroprocedures were observed in a few instances, most of the comparisons showed satisfactory agreement. The average difference was not significant for free cholesterol. The microprocedure gave a significantly higher average result for total cholesterol than the macromethod but the difference could be largely accounted for by small losses in one of the macroprocedures.

With a single exception the percentage of free in total cholesterol determined by the microprocedure differed from the corresponding macro value by less (usually much less) than the range of variation reported by the author in healthy human subjects.

It is concluded that the microprocedure gives essentially the same result as the macrogravimetric method and that the former is not subject to sufficiently large errors to account for the marked

discrepancy between findings in normal blood serum reported by the author (2) and those of other investigations.

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THE SYNTHESIS OF α-GLUTAMYLCYSTEINYLGLYCINE (ISOGLUTATHIONE)

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In investigations of the chemistry and physiological action of the γ-glutamyl peptide, glutathione, it would be of considerable value to have available the isomeric peptide, in which the cysteinylglycine portion of the molecule is attached to the α-carboxyl grouping of the glutamic acid, namely the α-glutamylcysteinylglycine. For convenience this compound might be designated as isoglutathione.

To have available such a compound would make possible interesting comparative studies of this isomer of glutathione with glutathione itself. Studies of the stabilities of these two compounds might throw some light on the rôle that the γ linkage plays in certain peculiar phases of the chemistry of glutathione, such as the ease of the splitting off of cysteinylglycine. Kendall, Mason, and McKenzie (1) found, for example, that merely heating glutathione in aqueous solution at 62° would readily cleave the compound yielding cysteinylglycine. Hopkins (2) also observed that when glutathione was heated in boiling water, pyrrolidonecarboxylic acid was split off with the formation of the diketopiperazine of cysteinylglycine. It would also be of interest to know whether the stability of the sulfur towards alkali and the oxidation-reduction behavior are influenced by these two types of linkages. A comparison of the behavior of the two isomers towards digestive enzymes would no doubt yield data of value. Furthermore, it would be interesting to compare their behavior in certain physiological reactions, particularly where it is believed that glutathione plays some specific rôle, such as coenzyme for methylglyoxalase.

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In some experiments, which we had carried out exploring various possibilities for the synthesis of glutathione, we attempted the opening of carbobenzoxyglutamic anhydride with S-benzylcystein-ylglycine in pyridine solution.   In this work we obtained indications that the anhydride opened in such a manner as to yield predominantly the derivative of the α-peptide.   Because of the value of having available the isomeric glutathione, we have prosecuted further these observations and have succeeded in isolating the isoglutathione in crystalline form.

The N-carbobenzoxy-α-glutamyl-S-benzylcysteinylglycine, resulting from the opening of the carbobenzoxyglutamic anhydride,

(a) (b)
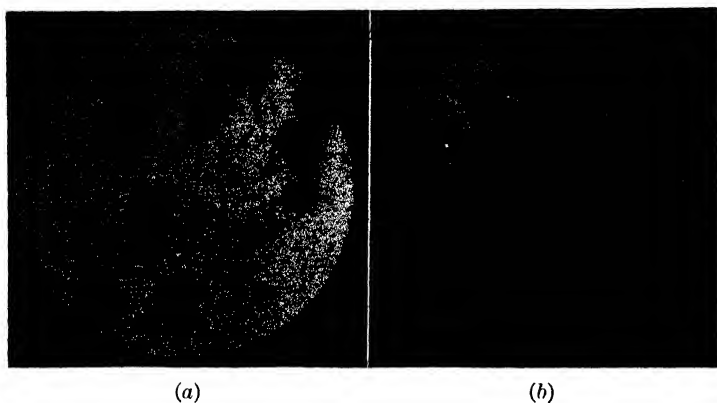
Fig. 1. (a) Isoglutathione ($\times$ 150); (b) glutathione ($\times$ 150)

was obtained in crystalline form and was reduced in liquid ammonia with metallic sodium.   The benzyl and carbobenzoxy groups were removed, as was expected from our experience with the reduction of dicarbobenzoxycystinyldiglycine and S-benzylcystein-ylglycine (3).   The tripeptide which resulted was isolated by way of its mercury and copper salts.   It is of interest to note that the latter salt did not possess the sheen which is so characteristic of the copper salt of glutathione.   The isoglutathione was regenerated from the copper salt in the usual manner and was obtained in the form of very thin platelets which appear almost as needles, as shown in Fig. 1, in which is also given the crystalline form of glutathione.   The compound melted with decomposition at 152–

153° and showed a specific rotation of $[\alpha]_D^{25} = +2.5°$ for a 2 per cent solution in water.   The analytical values agreed with the theoretical values.   However, to show beyond any question that the above opening of the anhydride really yielded the α-peptide, the N-carbobenzoxy-α-glutamyl-S-benzylcysteinylglycine was synthesized by another method which definitely placed the peptide linkage in the α position.   This method, however, was less convenient than the anhydride opening and was utilized only to confirm the presence of the α linkage.

This was accomplished by condensing γ-ethyl-N-carbobenzoxy-glutamyl chloride with S-benzylcysteinylglycine methyl ester. The γ-ethyl glutamic acid hydrochloride was prepared by the method of Bergmann and Zervas (4) and was converted into the carbobenzoxy compound.   The condensation product was saponified and the N-carbobenzoxy-α-glutamyl-S-benzylcysteinylglycine which resulted was obtained in crystalline form   This compound was found to be identical with the product obtained from the opening in pyridine of carbobenzoxyglutamic anhydride with S-benzylcysteinylglycine.

<div align="center">EXPERIMENTAL</div>

Preparation   of   N-Carbobenzoxy-α-Glutamyl-S-Benzylcysteinyl-glycine   with   Carbobenzoxyglutamic   Anhydride—13.4 gm. of S-benzylcysteinylglycine, prepared by the method of Loring and du Vigneaud (3), were powdered and were suspended in 200 cc. of anhydrous pyridine.   13.2 gm. of carbobenzoxyglutamic anhydride, prepared according to the directions of Bergmann and Zervas (5), were added in ten portions to the above suspension.   The mixture was shaken after each addition.   The carbobenzoxyglutamic anhydride together with the S-benzylcysteinylglycine gradually went into solution and the mixture became warm.   After standing for an hour at room temperature, the solution was separated from a small amount of insoluble material and was concentrated *in vacuo*. The residue was transferred to a beaker with the aid of 200 cc. of water and a few cc. of N NaOH.   The solution, which was very gelatinous, was acidified with dilute HCl and the amorphous precipitate which separated was filtered and washed thoroughly with water.   The moist filter cake was dissolved in about 50 cc. of warm dioxane and filtered.   The filtrate was diluted with a little

water and was placed in the refrigerator to crystallize. The product, which separated as very fine needles, was filtered and was washed with 50 per cent dioxane and then with ether. A second crop was obtained by concentration of the mother liquors. The yield was 11 gm., which represented 40 per cent of the theoretical amount. After recrystallization from dioxane, the compound melted at 191–192°. The analytical values of 7.98 per cent N and 6.02 per cent S correspond to the theoretical values of 7.91 per cent N and 6.03 per cent S for $C_{25}H_{29}O_8N_3S$.

Preparation of N-Carbobenzoxy-α-Glutamyl-S-Benzylcysteinylglycine with γ-Ethyl-N-Carbobenzoxyglutamic Acid—γ-Ethyl glutamic acid hydrochloride was prepared according to the directions of Bergmann and Zervas (4) and was converted to the carbobenzoxy compound with carbobenzoxy chloride and MgO. The carbobenzoxy derivative agreed in properties with the preparation of Abderhalden and Nienburg (6) which, however, as shown by Bergmann and Zervas (4), was mislabeled the α ester instead of the γ ester. 3 gm. of this compound were converted to the acid chloride and were condensed with 7.6 gm. of the free methyl ester of S-benzylcysteinylglycine, as in the corresponding step in the glutathione synthesis (7). 3.6 gm. of crude condensation product were obtained. 3 gm. of the material were dissolved in dioxane and were saponified with 13 cc. of N NaOH. There was obtained about 1 gm. of amorphous product, which, after two crystallizations from dioxane, yielded needles which melted at 191–192°. A mixture of this compound with a sample of the crystalline product obtained from the opening of the carbobenzoxyglutamic anhydride with S-benzylcysteinylglycine also melted at 191–192°.

Preparation of Isoglutathione—The free tripeptide was prepared from the N-carbobenzoxy-S-benzyl derivative by the procedure outlined by du Vigneaud and Miller in the synthesis of glutathione (7). 10 gm. of N-carbobenzoxy-α-glutamyl-S-benzylcysteinylglycine were dissolved in 200 cc. of dry liquid ammonia and reduced with about 2.5 gm. of metallic sodium. 7.3 gm. of $(NH_4)_2SO_4$ were added to neutralize the sodium and the ammonia was evaporated. The isoglutathione was precipitated first with $HgSO_4$ solution and then after the mercury salt was decomposed with H_2S, the peptide was reprecipitated with cuprous oxide. The final filtrate from the decomposed cuprous mercaptide was evap-

orated in a desiccator to a heavy syrup. The syrup crystallized as tiny platelets when the sides of the container were scratched. The crystallizing dish was placed in a desiccator filled with hydrogen and crystallization was allowed to proceed overnight in the refrigerator. The compound was taken up with the aid of a little 50 per cent alcohol and filtered. The crystals were washed with cold 50 per cent alcohol and finally with 95 per cent alcohol. After the product was dried, it amounted to 3 gm., which represented 50 per cent of the theoretical yield. A small sample of the product was recrystallized for analysis by dissolving in a minimum volume of water and diluting the solution with an equal volume of alcohol. After the solution had stood overnight in the refrigerator, the compound crystallized out and was filtered and dried. The peptide melted with decomposition at 152–153° and showed a specific rotation of $[\alpha]_D^{25} = +2.5°$ for a 2 per cent solution in water. When subjected to analysis, the compound showed the following composition.

$C_{10}H_{17}O_6N_3S \cdot H_2O$.   Calculated.   C 36.90, H 5.89, N 12.92, S 9.86
                          Found.   " 37.20, " 5.76, " 13.01, " 9.96

The authors wish to thank Mr. C. Rodden, microanalyst of this laboratory, for carrying out the microanalyses.

SUMMARY

A synthesis of α-glutamylcysteinylglycine, which has been designated isoglutathione, has been presented.

The possibilities for comparative studies of this compound with the isomeric glutathione have been pointed out.

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SPECTROPHOTOMETRIC STUDIES OF THE COLOR DEVELOPMENT IN THE ANALYSIS OF SUGAR BY THE BENEDICT METHOD AND OF CHOLESTEROL BY THE LIEBERMANN-BURCHARD REACTION*

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For colorimetric analyses either by visual reading or photoelectric cell, the advantages of employing light confined to selected zones of the spectrum are now recognized. Since many solutions used in biological colorimetric analyses display changes in tint and intensity of color developed over a period of time after preparation, the isolation in the spectrum of a zone of constant transmission may be more desirable than the isolation of a zone of maximal absorption. The selection of the optimal region in the spectrum for making measurements requires investigation for each colorimetric method. This paper presents an analysis of the color development during the first 30 to 60 minutes when sugar solutions are analyzed by the Benedict method (1) and cholesterol solutions are analyzed by utilizing the Liebermann-Burchard reaction; in both instances the color of the solutions obtained is unstable.

The development of color in the solutions studied was analyzed by the Razek-Mulder color analyzer.[1] This instrument is an automatic, photoelectric indicating and recording spectrophotometer by means of which a transmission curve of a given solution may be photographed over the visible range within 10 seconds. The rapidity with which individual curves may be obtained with this

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[1] Manufactured by the Thwing Instrument Company, Philadelphia.
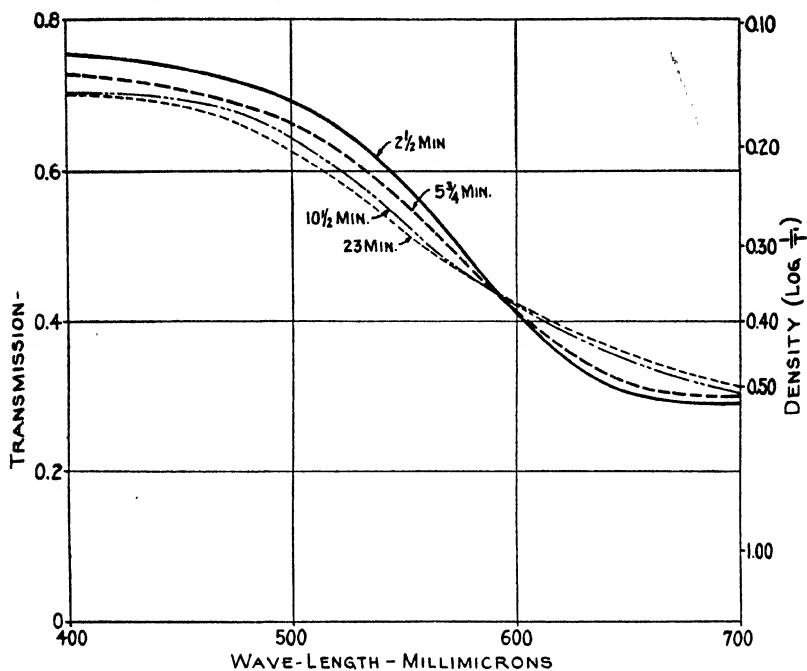
FIG. 1. Transmission curves of a tungstic acid filtrate of blood for sugar analysis by Benedict's method.
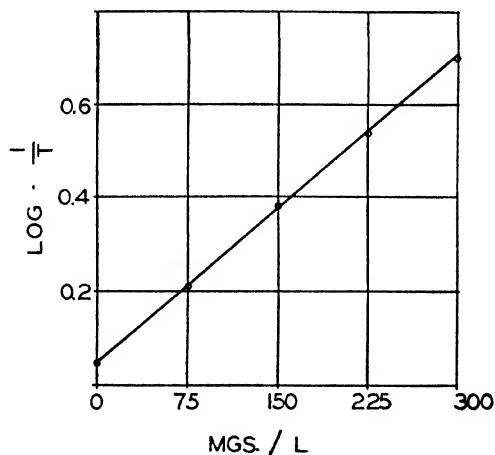
FIG. 2. Optical densities of sugar solutions at 592 $m\mu$ plotted against the concentrations.

instrument facilitates the study of solutions with changing color. Transmission curves for the solutions studied were obtained within 2 minutes after their preparation and subsequent curves at intervals up to 1 hour.   The transmission curves shown in Figs. 1 and 3 have been selected from the respective families of curves obtained at more frequent intervals of time.   Curves obtained at other intervals than those shown in Figs. 1 and 3 fall in series with the curves which are given.
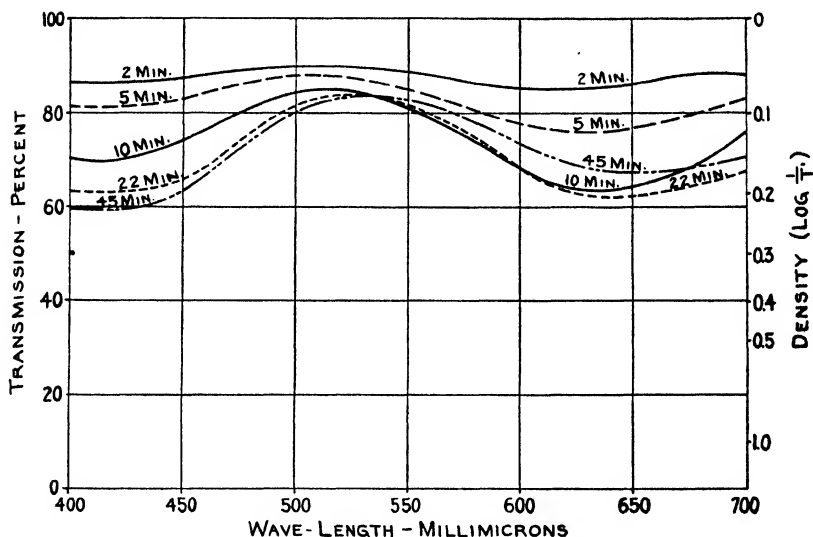
FIG. 3. Transmission curves of an alcohol-ether extract of blood serum by the Liebermann-Burchard reaction.

Transmission Curves of Sugar Solutions by Benedict's Method— Fig. 1 shows a series of transmission curves of a tungstic acid filtrate prepared from blood for sugar analysis by Benedict's method. During the 23 minutes of observation the percentage of transmission decreased in the blue range and increased in the red range of the spectrum.   In the blue range the increase in the optical density amounted to more than 20 per cent during this period. At a wave-length of approximately 592 $m\mu$ the curves crossed, indicating that the percentage of transmission at this wavelength was constant during the period of observation.   The point of intersection of the time curves for all of the sugar solutions we
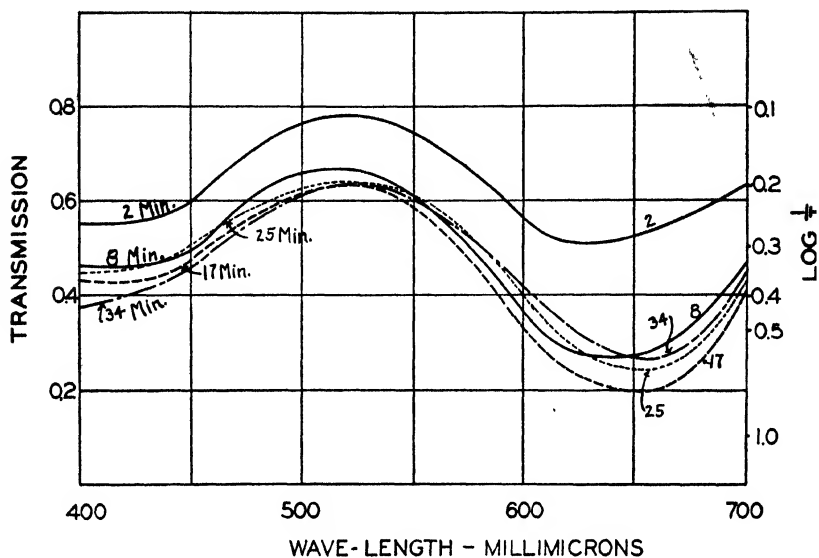
Fig. 4. Transmission curves of a cholesterol standard kept protected from light.
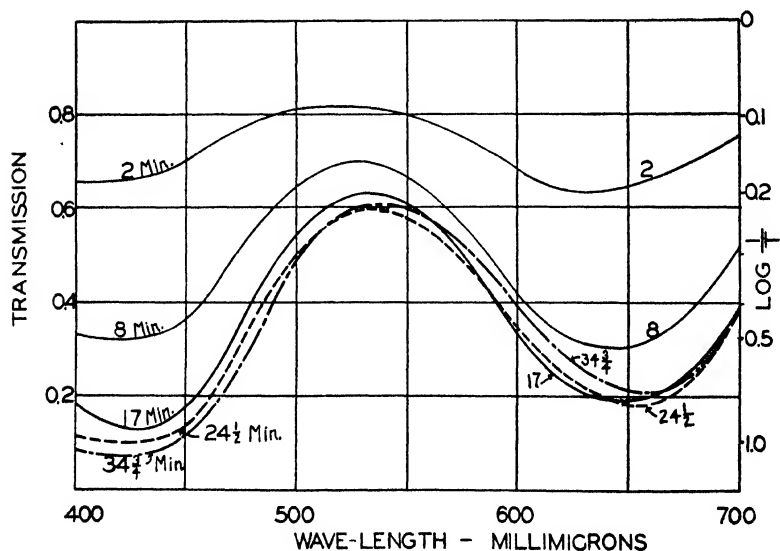
Fig. 5. Transmission curves of a cholesterol standard of the same concentration as shown in Fig. 4 exposed to daylight.

have analyzed by the Benedict method, whether from tungstic acid or zinc filtrates of blood or from aqueous glucose solutions of different concentrations, has always been located between 590 and 595 $m\mu$.

Fig. 2 shows that at 592 $m\mu$ the concentration of aqueous sugar solution was proportional to optical density over a 4-fold range of concentration.

Transmission Curves of Cholesterol Solutions by Liebermann-Burchard Reaction—Fig. 3 shows a series of transmission curves
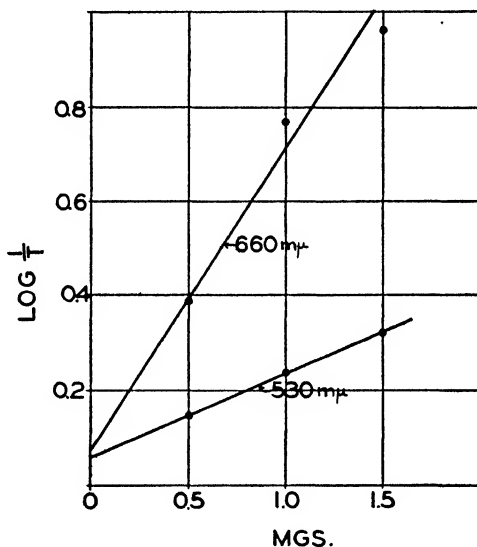
Fig. 6. Optical densities of cholesterol solutions at 530 $m\mu$ after 10 minutes and at 660 $m\mu$ after 20 minutes plotted against the concentrations.

during 35 minutes of the color development by the Liebermann-Burchard reaction with an alcohol-ether extract of blood serum according to the method of Bloor, Pelkan, and Allen (2). In the blue-violet region the percentage of transmission decreased throughout the period; in the orange-red region it decreased to a minimum at about 20 minutes, then increased during the remaining 15 minutes of the observation. Constancy of transmission was observed after 10 minutes at a wave-length of 530 $m\mu$.

It is known that the development of color in cholesterol solutions is influenced by exposure to light. In Figs. 4 and 5 are trans-

mission curves from two cholesterol standards of the same concentration.   The curves in Fig. 4 were obtained from the solution exposed to daylight throughout the period of study; the curves in Fig. 5, from the solution kept protected from light except for the intervals of 10 seconds each required to obtain each curve.   The solution kept in the dark developed a marked absorption in the blue-violet range not observed in the solution exposed to light. The transmission curves in the region from 550 to 700 $m\mu$ when obtained at corresponding intervals of time were approximately the same in both the exposed and unexposed solutions.

In Fig. 6 are plotted concentrations of cholesterol in solution against optical densities at 530 $m\mu$ after 10 minutes and at 660 $m\mu$ between 20 and 25 minutes.   At 530 $m\mu$ after 10 minutes the transmission was constant and optical densitiy was proportional to concentration.   At 660 $m\mu$ the transmission was not constant even after 20 minutes and the plot of optical density against concentration was curved.

<div align="center">DISCUSSION</div>

Although both the sugar and cholesterol solutions varied in color during the period of observation, nevertheless each type of solution exhibited constancy in transmission at a characteristic portion of the spectrum.   The standard method for determining transmission at a definite region in the spectrum is by means of a spectrophotometer.   However, the general availability of colorimeters of the Duboscq type makes it convenient to adapt the colorimeter for the determination of transmission in a selected spectral zone as near the optimal as the limitations of the colorimeter permit.

Approximate isolation of the region between 590 and 595 $m\mu$ for the measurement of sugar solutions by the Benedict method may be accomplished either by placing appropriate filters[2] in the lens system and illuminating the colorimeter by an incandescent lamp or by using the yellow line in the spectrum of a mercury or helium

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[2] A combination of either three Wratten filters (Nos. 24-A, 24-A, 53) or two Corning Glass filters (No. 401, 4 mm. thickness, and No. 246, 125 per cent) placed in the lens system of the colorimeter is suitable for readings in this region.   These combinations are very dense, so that illumination of the system must be greatly increased.

lamp[3] (579 and 587 $m\mu$ respectively).   Photometric measurements
may be made against a standard consisting of a neutral tint filter
of appropriate optical density.[4]

Fig. 7 is a photograph of a type of mercury or helium lamp
operated from a high voltage transformer which is adapted for use
with colorimeters.   When measurements are being made con-
tinuously, the helium lamp, because of the heat it generates, is
less desirable than the mercury lamp.   Colorimetric measure-
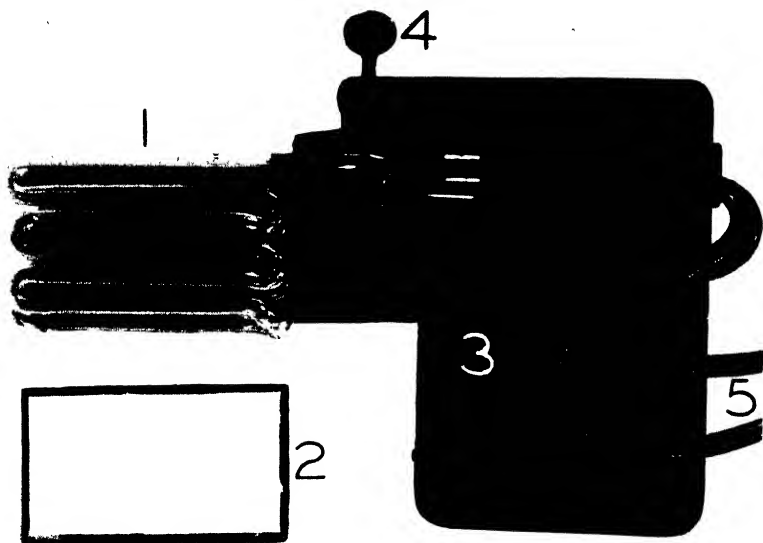
FIG. 7. Mercury or helium lamp.   *1*, Pyrex tubing arranged in two layers
to provide a continuous sheet of light; *2*, removable opal glass reflector
for insertion beneath tubing; *3*, cast iron base; *4*, thumbscrew for adjust-
ment to colorimeter; *5*, cable to high voltage transformer.

ments of sugar solutions in which light is limited by any of these
methods to the region of the spectrum desired are suitably con-
stant during the interval from 10 to 60 minutes after preparation

[3] Isolation of either of these lines is secured by placing a No. 4 mono-
chromatic Corning Glass filter over the eyepiece of the colorimeter.

[4] NG-3, NG-4, and NG-5 Jena glass filters may be used for this purpose.
Satisfactory neutral gray filters may also be provided by using monel
metal gauze of various meshes.

of the solution.   Reproducible results are obtained by different observers after 10 minutes to within 0.1 mm. when the depth of solution is approximately 10 mm.

With the sugar solutions the transmissions in the region beyond 650 $m\mu$ did not change appreciably after 20 minutes.   Dr. C. F. Park, working in our laboratory, has preferred to make his readings through a deep red filter 20 minutes or more after the final preparation of the solutions.

With cholesterol solutions the region of most constant transmission was at 530 $m\mu$ after 10 minutes.   However, in this zone absorption is minimal, so that the procedure of analysis would require change either by increasing the concentration of cholesterol in the final solution several fold or by increasing the depth of solution examined in the colorimeter.   As a routine procedure neither of these changes is convenient.   For our analyses we have preferred to adhere to the usual concentrations and to make the readings in the red zone centering about 650 $m\mu$ promptly after 20 minutes, when absorption is nearly maximal.[5]   Obviously because of the changing absorption we use a cholesterol standard of approximately the same concentration as the unknown rather than a constant, neutral tint standard.   The curves in Figs. 4 and 5 indicate that when measurements are made in this zone it is unnecessary to protect the solutions from light.

SUMMARY

The development of color in sugar solutions by the Benedict method and in cholesterol solutions by the Liebermann-Burchard reaction was studied by means of a photoelectric spectrophotometer which recorded within 10 seconds the transmission at each wave-length throughout the visible range.   The first curve was obtained 2 minutes after preparation of a given solution and subsequent curves at intervals up to 1 hour.   These studies afford a basis for selecting the optimal spectral zone for colorimetry in these two methods.

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[5] A Wratten No. 71-A filter, as suggested by Schoenheimer and Sperry (3), has proved satisfactory.

ON PROTEOLYTIC ENZYMES

XIII. SYNTHETIC SUBSTRATES FOR CHYMOTRYPSIN

By MAX BERGMANN and JOSEPH S. FRUTON

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It was found that a preparation of once recrystallized chymotrypsin (1) splits carbobenzoxyglycyl-*l*-tyrosylglycineamide with great rapidity. A sample of four times recrystallized chymotrypsin, obtained from Dr. J. H. Northrop, split this substrate with equal rapidity. The hydrolysis of the peptide derivative is therefore a property of the crystalline enzyme itself and is not attributable to an impurity in the crude product.

In the splitting of the substrate one peptide linkage was hydrolyzed with the formation of carbobenzoxyglycyl-*l*-tyrosine. If crystalline carboxypeptidase (2) was added to the hydrolysate, this reaction product was split further to yield free tyrosine. Thus, if carbobenzoxyglycyl-*l*-tyrosylglycineamide had appeared in the human or animal intestine, it would have undergone, in analogy with the fate of a true protein, a successive digestion by the enzymes of the intestinal contents followed by the absorption of a portion of the split-products.

Other compounds, closely related to carbobenzoxyglycyl-*l*-tyrosylglycineamide were tested for lability in the presence of chymotrypsin. Thus, N-carbobenzoxy-*l*-tyrosylglycineamide and glycyl-*l*-tyrosylglycineamide are split very easily by the enzyme; carbobenzoxyglycyl-*l*-phenylalanylglycineamide and N-carbobenzoxy-*l*-tyrosylglycylglycineamide, much more slowly (Table I). The following are not attacked by the enzyme: N-carbobenzoxy-*l*-tyrosylglycine, carbobenzoxyglycyl-*l*-glutamylglycineamide, benzoylglycyl-*l*-lysineamide, carbobenzoxyglycyl-*l*-leucylglycineamide, benzoyl-*l*-leucyl-*l*-leucylglycine, and chloroacetyltyrosine.

Until recently, there were no synthetic peptides available which

TABLE I

Behavior of Synthetic Substrates toward Chymotrypsin

| Enzyme | Substrate | Time | Hydrolysis | | Isolation of products |
			Van Slyke	Titration	
		hrs.	*per cent*	*per cent*	
Chymotrypsin, once recrystallized, 0.65 mg. of protein N per cc.	Carbobenzoxyglycyl-*l*-tyrosylglycineamide	4	90	93	Carbobenzoxyglycyl-*l*-tyrosine
		22	103	98	
	Glycyl-*l*-tyrosylglycineamide	0.5	55	61	
		17	96	95	
	Carbobenzoxyglycyl-*l*-phenylalanylglycineamide	4	23	23	
		22	40	42	
	Carbobenzoxy-*l*-tyrosylglycineamide	4	89	93	Carbobenzoxy-*l*-tyrosine
		24	96	98	
	Carbobenzoxy-*l*-tyrosylglycine	24	0	1	
	Carbobenzoxy-*l*-tyrosylglycylglycineamide	4	8	5	
		20	17	19	
	Benzoylglycyl-*l*-lysineamide	6		4	
		24		4	
	Carbobenzoxyglycyl-*l*-glutamylglycineamide	20		0	
	Carbobenzoxyglycyl-*l*-leucylglycineamide	22		1	
	Benzoyl-*l*-leucyl-*l*-leucylglycine	20		0	
	Chloroacetyl-*l*-tyrosine	18		1	
	Gelatin	2		0.22*	
		18		0.59*	
	Casein	3	0.49†		
		5	0.63†		
Chymotrypsin, 4 times recrystallized, 0.7 mg. of protein N per cc.	Carbobenzoxyglycyl-*l*-tyrosylglycineamide	3		88	
		20		101	
	Casein	3	0.62†		
		5	0.76†		

* Increase in titer of 0.01 N KOH for 8 mg. of gelatin.

† Increase in mg. of amino nitrogen for 67 mg. of casein.

had been shown to be hydrolyzed by a proteinase, and no peptidase was known which would attack genuine proteins. These factors led to the possibility that in proteins there were present some unknown types of linkage which could be split by the proteinases.[1] This uncertainty may now be set aside. Chymotrypsin is a peptidase just as are Cathepsin I, Papain I, and Bromelin I. All these four proteinases belong to that group of peptidases which do not require an α-amino group or an α-carboxyl as a point of enzyme attachment. On the contrary, several of these proteinases are incapable of operating in the vicinity of an α-carboxyl, others in the vicinity of an α-amino group. Thus, they split their substrates preferably, although not exclusively, at interior peptide linkages; they are endopeptidases (7).[2]

The opinion has been widely held (9) that proteinases require basic or acidic groups or phenolic tyrosine hydroxyls of the substrate side chains at points of attachment. This assumption has not been found valid in the case of chymotrypsin, or for Papain I, Cathepsin I, and Bromelin I. · The proteinases are particularly sensitive to the presence and the special nature of the side chains in the substrate; for example, papain distinguishes between glycyl and leucyl (4), and chymotrypsin between phenylalanyl and leucyl. However, this faculty of differentiation cannot depend on a combination of the enzyme with the side chain of the substrate, but may rather be attributed to the effect which the presence or absence of the various side chains may have on the sensitivity of the peptide linkages to the action of the enzymes. This conclusion seems to hold at least for the four proteinases which have been investigated for their action on synthetic substrates. However, the splitting of a peptide linkage by a proteinase is not only affected by the two amino acid residues which directly participate in the peptide bond but by more distant groups as well; this effect is clearly visible in the decrease of splitting of the tyrosyl-

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[1] The claim of several investigators (3) that diketopiperazines with basic or acidic side chains were substrates for proteinases has not been confirmed. *Cf.* Bergmann, Zervas, and Fruton (4), Greenstein (5), and Waldschmidt-Leitz and Gärtner (6).

[2] It may be mentioned that Linderstrøm-Lang has expressed the view that proteinases are "enzymes which attack the peptide linkages in the middle of the chain" (8).

glycine linkage by chymotrypsin on passing from carbobenzoxy-tyrosylglycineamide to carbobenzoxytyrosylglycylglycineamide.

The influence of the side chains varies in extent with the different proteinases. The only peptide linkages in synthetic compounds which have been split thus far by chymotrypsin were those in which the tyrosine or phenylalanine supplies the peptide carbonyl. This may be contrasted with Papain I which has been found to split peptide linkages where the peptide carbonyl belongs to glycine, leucine, glutamic acid, or lysine, and where the peptide imino group belongs to glycine, leucine, glutamic acid, lysine, or tyrosine.

The great range of action of Papain I results in a successive splitting in some of the synthetic substrates at several peptide linkages. An illustration of this behavior is found in the hydrolysis of carbobenzoxyglycyltyrosylglycineamide at two peptide linkages to yield carbobenzoxyglycine, tyrosylglycine, and ammonia (Table II). It will be noted that Papain I and chymotrypsin respectively attack this substrate at entirely different positions in the molecule.

The finding of various simple synthetic substrates for proteinases, as reported in the present and in earlier papers, has opened the possibility of differentiating the proteinases on the basis of differences in their specificity toward the various synthetic substrates. The comparative behavior of papain and chymotrypsin toward carbobenzoxyglycyltyrosylglycineamide indicates the possibility of differentiating two proteinases by the determination of the points of attack within the same synthetic substrate.

Carbobenzoxyglycyltyrosylglycineamide seems to be split very slowly by pepsin (Table II). Experiments are now in progress to test the possibility of developing a suitable substrate for pepsin by the systematic alteration of the above substance.

It was recently reported from this laboratory (10) that "tryptic proteinase" splits benzoylglycyl-l-lysineamide quite rapidly at two peptide linkages. It is to be noted that tryptic proteinase, prepared according to Waldschmidt-Leitz and Purr (11), is a mixture of several enzymes (12). It was therefore desirable to determine the identity of the component responsible for the splitting of the above compound. Chymotrypsin is ineffective (Table I). A

solution of once recrystallized trypsin obtained from Dr. J. H. Northrop and kept over a year at 6° showed a splitting which stopped at 50 per cent after 3 hours at pH 7.8 (Table II). A fresh preparation of thrice recrystallized trypsin sent to us by Dr. Northrop was inactive towards benzoylglycyl-*l*-lysineamide, as was a

TABLE II

Behavior of Synthetic Substrates toward Proteinases

Enzyme	Substrate	Time	Hydrolysis		Isolation of products
			Van Slyke	Titration	
		hrs.	*per cent*	*per cent*	
Trypsin, 3 times recrystallized, 0.65 mg. of protein N per cc.	Benzoylglycyl-*l*-lysineamide	24		2	
		48		3	
	Carbobenzoxyglycyl-*l*-tyrosylglycineamide	22		0	
	Casein	3	0.17*		
		5	0.26*		
Trypsin, once recrystallized, 0.4 mg. of protein N per cc.	Benzoylglycyllysineamide	3		50	
		20		52	
Papain-HCN	Carbobenzoxyglycyl-*l*-tyrosylglycineamide	3		94	Carbobenzoxyglycine
		22		202	Tyrosylglycine
		50		201	
Pepsin, 10 mg. 1:14,000 per cc.	"	26	7.0		
		48	11.0		
	Carbobenzoxyglycyl-*l*-glutamylglycineamide	20	0.0		
		44	3.0		

* Increase in mg. of amino nitrogen for 67 mg. of casein.

mixture of this enzyme preparation with chymotrypsin. These findings lead to the conclusion that in cattle pancreas and in "tryptic proteinase" there exists a proteinase which is different from trypsin and chymotrypsin. This enzyme, which splits benzoylglycyllysineamide, may be designated as heterotrypsin.

The authors wish to express their thanks to Dr. J. H. Northrop, Dr. P. A. Levene, and Dr. G. Meyer for placing at their disposal samples of crystalline trypsin and chymotrypsin.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Carbobenzoxyglycyl-l-Tyrosylglycineamide*</div>

N-Carbobenzoxy -o-Acetyl-l-Tyrosylglycine Ethyl Ester—13 gm. of N-carbobenzoxy-*o*-acetyl-*l*-tyrosyl chloride (13) were added to a dry ether solution of glycine ethyl ester (from 25 gm. of the hydrochloride). After standing 1 hour at room temperature, the reaction mixture was filtered and the filtrate successively extracted with hydrochloric acid, bicarbonate, and water, dried, and evaporated down. The residue (10 gm.) was recrystallized from ethyl acetate. M.p., 127°.

<div align="center">

$C_{23}H_{26}O_7N_2$.   Calculated.   C 62.4, H 5.9, N 6.3
442.2   Found.   " 62.5, " 5.9, " 6.3

</div>

Carbobenzoxyglycyl-l-Tyrosylglycincamide—4.5 gm. of the above ester were hydrogenated catalytically in methanol containing 1 cc. of 10 N hydrochloric acid. The hydrogenation mixture was filtered and the filtrate evaporated down, yielding a syrup which was dissolved in 6 cc. of water and converted to the free ester in the usual manner with ethyl acetate as the organic solvent. After drying briefly over Na_2SO_4, there were added 1.3 gm. of carbobenzoxyglycyl chloride, and after some shaking, another portion of 1.3 gm. of carbobenzoxyglycyl chloride was added together with 10 cc. of 10 per cent potassium bicarbonate solution. Finally, an additional 20 cc. of bicarbonate solution were added, the aqueous layer was separated off, and the ethyl acetate layer washed with water, dilute hydrochloric acid, bicarbonate, and water, and then dried and evaporated down. The resulting syrup was dissolved in 25 cc. of methanol saturated with ammonia at 0° and allowed to stand at room temperature for 2 days. Upon evaporation a crystalline residue resulted. Yield, 2.5 gm. After recrystallization from hot water, the substance melted at 192°.

<div align="center">

$C_{21}H_{24}O_5N_4$.   Calculated.   C 58.9, H 5.5, N 13.1
428.2   Found.   " 58.9, " 5.6, " 13.1

</div>

The enzymatic hydrolysate by chymotrypsin of 595 mg. of this substance was filtered, concentrated to a small volume, acidified to

Congo red, and the resulting oil extracted with ethyl acetate. The split-product was purified by passage through bicarbonate, precipitation by acid, and reextraction by ethyl acetate. On concentration of the ethyl acetate solution, 350 mg. (68 per cent of the theory) of a crystalline material were obtained, having a melting point of 105°. The mixed melting point with carbo-benzoxyglycyl-*l*-tyrosine was 105–106°.

$C_{19}H_{20}O_6N_2$ (372.2). Calculated, N 7.5; found, N 7.4

The chymotrypsin hydrolysate of 53 mg. of the above substance was filtered and to it were added 5 mg. of crystalline carboxypeptidase. After 1 hour at 40° the titer of the solution had increased by an amount of 0.01 N alkali equivalent to 30 per cent of one peptide linkage, and tyrosine crystals separated out.

The enzymatic hydrolysate by papain-HCN of 400 mg. of carbobenzoxyglycyltyrosylglycineamide was evaporated down to a small volume, acidified to Congo red, and the resulting oil extracted with ethyl acetate. The ethyl acetate layer was then extracted with bicarbonate solution and the aqueous layer acidified, yielding 154 mg. of a crystalline precipitate (75 per cent of the theory). M. p., 120°. The mixed melting point with carbobenzoxyglycine was 120°.

$C_{10}H_{11}O_4N$ (209.1). Calculated, N 6.7; found, N 6.7

The acid aqueous layer from the ethyl acetate extraction was neutralized, filtered, and treated with 0.25 cc. of carbobenzoxy chloride and 2.5 cc. of N NaOH. When the product was acidified to Congo red, an oil was obtained which crystallized after a short period in the ice box. Yield, 238 mg. (64 per cent of the theory). After recrystallization from ethyl acetate-petroleum ether, the melting point was 96°. The mixed melting point with carbobenzoxy-*l*-tyrosylglycine was 96°.

$C_{19}H_{20}O_6N_2$ (372.2). Calculated, N 7.5; found, N 7.7

Glycyltyrosylglycineamide Hydrochloride—0.5 gm. of the carbo-benzoxy compound was hydrogenated catalytically in the presence of 0.15 cc. of concentrated hydrochloric acid. When the filtrate was evaporated down from the hydrogenation mixture, a crystalline substance was obtained. M.p., 89–90°.

$C_{13}H_{18}O_4N_4 \cdot HCl \cdot H_2O$. Calculated. C 45.0, H 6.1, N 16.3
Found. " 45.3, " 6.0, " 16.3

Carbobenzoxy-l-Tyrosylglycineamide—1 gm. of N-carbobenzoxy-*o*-acetyl-*l*-tyrosylglycine ethyl ester was dissolved in methanol saturated with ammonia and left at room temperature for 2 days. The solution was then filtered, evaporated down, and the resulting syrup taken up in a small volume of methanol.   On careful addition of water an oil resulted, which crystallized on standing overnight in the cold.   Yield, 0.7 gm.   After recrystallization from methanol-water the substance melted at 116°.

$C_{19}H_{21}O_5N_3$.   Calculated.   C 61.4, H 5.7, N 11.3
371.2   Found.   " 61.4, " 5.8, " 11.6

The enzymatic hydrolysate by chymotrypsin of 350 mg. of this substance was filtered, concentrated to a small volume, and acidified to Congo red.   The resulting oil was extracted with ethyl acetate, which in turn was extracted with bicarbonate solution. When the aqueous layer was acidified, an oil appeared which crystallized after cooling.   Yield, 265 mg. (80 per cent of the theory).

N-Carbobenzoxytyrosine—
$C_{17}H_{17}O_5N·H_2O$.   Calculated.   C 61.6, H 5.8, N 4.1
333.2   Found.   " 61.8, " 5.7, " 4.0

Carbobenzoxy-l-Tyrosylglycine—2.8 gm. of N-carbobenzoxy-*o*-acetyl-*l*-tyrosylglycine ethyl ester were dissolved in 20 cc. of methyl alcohol, and 13 cc. of N NaOH were added.   After standing at room temperature for 20 minutes, the solution was acidified with 15 cc. of normal hydrochloric acid.   The resulting crystals were recrystallized from methanol-water.   Yield, 2.1 gm.   M.p., 100°.

$C_{19}H_{20}O_6N_2$.   Calculated.   C 61.3, H 5.4, N 7.5
372.2   Found.   " 61.4, " 5.7, " 7.6

Carbobenzoxyglycyl-l-Tyrosine Ethyl Ester—2.5 gm. of carbobenzoxyglycyl chloride were added to an ethyl acetate solution of tyrosine ethyl ester (from 8 gm. of the hydrochloride).   The reaction mixture was allowed to stand for 2 hours, the tyrosine ethyl ester hydrochloride was filtered off, and the filtrate was washed with dilute hydrochloric acid, bicarbonate, and water,

dried, and evaporated down. The resulting crystals were recrystallized from ethyl acetate. Yield, 2.3 gm. M.p., 118°.

$C_{21}H_{24}O_6N_2$.   Calculated.   C 63.0, H 6.1, N 7.0
400.2   Found.   " 62.7, " 6.2, " 6.8

Carbobenzoxyglycyl-l-Tyrosine—1 gm. of the ester was shaken with 7 cc. of N NaOH and after the solution was allowed to stand at room temperature for 15 minutes, it was acidified with N hydrochloric acid. The resulting oil was taken up in ethyl acetate and transferred to bicarbonate. When the alkaline solution was acidified, the free acid crystallized. After recrystallization from ethyl acetate, the melting point was 107°.

$C_{19}H_{20}O_6N_2$.   Calculated.   C 61.3, H 5.4, N 7.5
372.2   Found.   " 61.4, " 5.4, " 7.4

Carbobenzoxy-l-Tyrosylglycylglycineamide

Carbobenzoxy-l-Tyrosylhydrazide—7.2 gm. of carbobenzoxy-*l*-tyrosine ethyl ester were dissolved in 30 cc. of hot ethyl alcohol and 2 cc. of hydrazine hydrate were added. The hydrazide crystallized out on standing at room temperature for 24 hours. Yield, 4.5 gm. M.p., 220°.

$C_{17}H_{19}O_4N_3$.   Calculated.   C 62.0, H 5.9, N 12.8
329.2   Found.   " 62.1, " 6.0, " 12.6

Carbobenzoxy-l-Tyrosylglycylglycine Ethyl Ester—3 gm. of carbobenzoxytyrosylhydrazide were suspended in 50 cc. of water and dissolved with the aid of 13 cc. of concentrated hydrochloric acid. With cooling and shaking there was added an aqueous solution of 0.7 gm. of sodium nitrite. The azide was extracted with ethyl acetate, washed with cold water, bicarbonate, and water, and then added to an ethyl acetate solution of glycylglycine ethyl ester (from 2.6 gm. of the hydrochloride). After standing overnight, the reaction mixture was extracted with dilute hydrochloric acid, bicarbonate, and water, dried, and evaporated down, yielding 2.5 gm. of a crystalline precipitate. M.p., 165°.

$C_{23}H_{27}O_7N_3$.   Calculated.   C 60.5, H 6.0, N 9.2
457.3   Found.   " 60.3, " 6.1, " 8.9

Carbobenzoxy-l-Tyrosylglycylglycineamide—1 gm. of the ester was dissolved in 20 cc. of methyl alcohol saturated with ammonia at 0°. After the mixture stood overnight at room temperature, the amide crystallized out. The substance was recrystallized from methyl alcohol. Yield, 0.8 gm. M.p., 218°.

$C_{21}H_{24}O_6N_4$. Calculated. C 58.9, H 5.5, N 13.1
428.2   Found.   " 59.1, " 5.7, " 12.9

Carbobenzoxyglycyl-l-Phenylalanylglycineamide

Carbobenzoxy-l-Phenylalanylglycine Ethyl Ester—4.2 gm. of carbobenzoxy-l-phenylalanyl chloride (14) were added to a dry ether solution of glycine ethyl ester (from 7.5 gm. of the hydrochloride). The reaction mixture was allowed to stand 1 hour at room temperature and was then filtered and washed successively with dilute hydrochloric acid, bicarbonate, and water, and dried. Upon evaporation of the solution, the substance began to crystallize out. Yield, 3.4 gm. M.p., 111°.

$C_{21}H_{24}O_5N_2$. Calculated. C 65.5, H 6.3, N 7.3
384.2   Found.   " 65.6, " 6.4, " 7.0

Carbobenzoxyglycyl-l-Phenylalanylglycineamide—3 gm. of the above compound were hydrogenated catalytically in methanol in the presence of 0.75 cc. of concentrated HCl. An ethyl acetate solution of the free ester was prepared from the resulting hydrochloride in the usual manner and to it were added, in two portions, 1.5 gm. of carbobenzoxyglycyl chloride; with the second portion there were added 5 cc. of 10 per cent potassium bicarbonate solution. The reaction mixture was shaken and cooled throughout. The ethyl acetate layer was washed with dilute hydrochloric acid, bicarbonate, and water, dried, and concentrated under diminished pressure. The resulting syrup was dissolved in methanol previously saturated with ammonia and left at room temperature for 2 days. On evaporation, a crystalline residue was obtained. Yield, 1.4 gm. The substance was recrystallized from hot water. M.p., 178°.

$C_{21}H_{24}O_5N_4$. Calculated. C 61.1, H 5.9, N 13.6
412.2   Found.   " 60.9, " 6.0, " 13.5

Carbobenzoxyglycyl-l-Glutamylglycineamide—2.5 gm. of the ester (4) were dissolved in 35 cc. of methanol previously saturated with

ammonia and allowed to stand at room temperature for 2 days. The syrup obtained on evaporation was taken up in water. When the aqueous solution was acidified, an oil separated out which crystallized on cooling. Yield, 1.7 gm. M.p., 175°.

$C_{17}H_{22}O_7N_4$.   Calculated.   C 51.8, H 5.9, N 14.2
394.2   Found.   " 52.0, " 5.8, " 13.9

Benzoylglycyl-l-Lysineamide—This substance was prepared as previously described (10).

Enzymatic Studies

The concentration of the synthetic substrates was 0.05 mM per cc. in all cases. The solutions were buffered by M/15 phosphate buffers at pH 7.6 to 7.8 for chymotrypsin and trypsin, and by 0.04 M citrate buffers at pH 5.0 for papain-HCN. The temperature in all cases was 40°. The liberated carboxyl groups were measured by the method of Grassmann and Heyde (15), and the amino groups determined by means of the Van Slyke apparatus. Enzyme blanks and controls to test the lability of the substrates in the absence of the enzymes were performed.

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THE SEPARATION OF CHOLINE AND ETHANOLAMINE*

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The separation of choline and ethanolamine or similar bases is a problem often encountered in work on the composition of phosphatides and other naturally occurring substances (*cf.* the reviews (1, 2)). Methods of separation have been described by Thierfelder and Schulze (3), Levene and Ingvaldsen (4), and Fourneau and González (5). They are mainly based on the different solubilities of choline and ethanolamine in various solvents (3, 4). The use of naphthalene sulfochloride also has been recommended (5).

An excellent reagent for the separation of choline from substances containing free amino groups has been found in carbobenzoxy chloride, $C_6H_5CH_2OCOCl$, which, as is well known, has been so successfully employed for the synthesis of peptides by Bergmann and Zervas (6). Whereas choline does not react with the reagent, a crystalline carbobenzoxy derivative of ethanolamine is formed in good yield from which the free base can be easily recovered. The advantages of this method are its rapidity, the formation of derivatives of definite melting point, and the ease with which recovery of the free bases can be effected. The last point will be especially valuable when mixtures of unknown bases are examined.

The same principle of separation is obviously applicable to many other substances containing free amino groups. One further instance, the isolation of glucosamine, is described in the following paper (7). It is hoped that other applications will be reported at a later date.

Isolation of Ethanolamine—To a solution of 1.94 gm. of choline hydrochloride and 1.70 gm. of ethanolamine hydrochloride in 9 cc. of water, 4.7 gm. of sodium bicarbonate and 10 cc. of chloroform were added. The mixture then was shaken with 4 gm. of carbobenzoxy chloride (6) which was added in four portions. It is advisable to cool the reaction mixture in running water for the first 15 minutes. After 1 hour no more CO_2 was given off. The mixture was acidified with dilute hydrochloric acid, more chloroform was added, and the two layers were separated. The aqueous layer was washed four times with chloroform. The united chloroform extracts were washed four times with very dilute hydrochloric acid and twice with water, dried with Na_2SO_4, and concentrated *in vacuo*. The remaining crude oil, weighing 4.2 gm., was dissolved in 5 cc. of warm chloroform, and petroleum ether (b.p. 30–60°) was added until a turbidity developed. After 12 hours in the refrigerator beautiful long needles had crystallized which weighed 1.98 gm. and melted at 65.5–66°. On addition of petroleum ether 0.45 gm. of the same compound was obtained from the mother liquor. The total yield of 2.43 gm. corresponds to 72 per cent of the theory. After one more crystallization from chloroform-petroleum ether, *carbobenzoxy ethanolamine*, $C_6H_5CH_2OCONHCH_2CH_2OH$, was obtained in long, colorless needles melting at 66.5°. There was no depression of the melting point on admixture of a sample of the carbobenzoxy derivative obtained from ethanolamine in the absence of choline.

Analysis—$C_{10}H_{13}O_3N$.   Calculated.   C 61.5, H 6.7, N 7.2
          195.1   Found.   " 61.7, " 6.8, " 7.0

In order to recover the free base 0.5 gm. of the carbobenzoxy compound was suspended in 5 cc. of N HCl and treated with hydrogen in presence of the palladium catalyst, according to Bergmann and Zervas (6), for 2 hours, when all had gone into solution. The syrup obtained on concentration *in vacuo* of the filtered solution was taken up in 0.8 cc. of concentrated hydrochloric acid, and a concentrated solution of gold chloride in concentrated hydrochloric acid was added. The gold chloride salt of ethanolamine crystallized immediately in yellow needles, which were filtered off, washed with chilled concentrated hydro-

chloric acid, and dried. The substance weighed 0.85 gm. (80 per cent of the theory), melted at 194–195° (corrected), and was identical with the gold salt obtained from pure ethanolamine.

Analysis—$C_2H_7ON \cdot HAuCl_4$ (401.1). Calculated, Au 49.2; found, Au 49.1

Isolation of Choline—The aqueous layer containing the choline was diluted with water to exactly 200 cc., and aliquot parts were used for the estimation of choline and for the preparation of derivatives. According to the microestimation method of Roman (8), which makes use of the formation of the enneaiodide of choline, $C_5H_{14}NOI_9$ (9), a total of 1.75 gm. of choline chloride was found, corresponding to 90.2 per cent of the choline chloride originally present. From another portion of the aqueous solution the gold chloride salt of choline was prepared as fine yellow platelets melting at 274° (corrected). The yield of the gold derivative corresponded to a total of 1.6 gm. of choline chloride.

Analysis—$C_5H_{14}NOCl \cdot AuCl_3$. Calculated. C 13.5, H 3.2, Au 44.5
443.1   Found.   " 13.6, " 3.2, " 44.4

The choline was further characterized by the formation of the typical chloroplatinate and by the formation of nitrocholine perchlorate, $C_3H_9N(ClO_4) \cdot CH_2CH_2ONO_2$, as platelets melting at 187° (corrected), which was prepared according to Hofmann and Höbold (10).

SUMMARY

A method is described for the separation of choline from ethanolamine by means of the carbobenzoxy derivative of the latter base.

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A METHOD FOR THE ISOLATION OF GLUCOSAMINE*

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Work on the amino sugars occurring in nature is hampered by the fact that the number of methods available for the isolation of these substances is very small. Among the derivatives of glucosamine which have been proposed for this purpose, one could mention the compounds obtained on condensation of glucqsamine with phenyl isocyanate (1) and naphthyl isocyanate (2), the Schiff bases obtained with salicylaldehyde (3) or anisaldehyde (4), the carbamino compound resulting from treatment of glucosamine with carbon dioxide and barium hydroxide (5), and the glucosamine salt of sozoiodolic acid (6). While most of these methods seem to give adequate results when applied to pure glucosamine, their use for the isolation of amino sugars from a mixture containing, for instance, simple sugars and amino acids is unattractive, as some of the reagents used are not easily removed from the reaction mixture or may react with other substances present as well. Therefore, the method still most widely used, when amino sugars are to be isolated from a hydrolysate, consists in decomposing the simple sugars present by means of strong hydrochloric acid, whereupon an attempt is made to isolate the amino sugar hydrochloride. This procedure obviously cannot be used when small amounts of material only are available for a total analysis.

The fact that amino sugars apparently occur in a number of substances of great biological importance, as, for example, in certain bacterial antigens or haptens, in the specific blood group

substances, in heparin, etc., prompted us to develop a procedure for the separation of glucosamine from simple sugars and amino acids.

The method consists principally in treating the mixture containing amino sugars, simple sugars, or amino acids in sodium bicarbonate solution with carbobenzoxy chloride, $C_6H_5CH_2OCOCl$, according to the method of Bergmann and Zervas (7). Under these conditions only the free amino groups react with the reagent. Carbobenzoxy glucosamine

$$\overset{\cdot}{C}H_2OH\cdot(CHOH)_3\cdot CHNH\cdot CHO$$
$$| $$
$$CO\cdot OCH_2C_6H_5$$
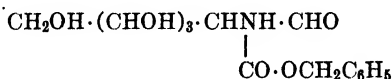
precipitates in crystalline form. The amino sugar may be regenerated from this compound by treatment with palladium and hydrogen (7). The amino acids are obtained as carbobenzoxy compounds on acidification of the filtrate, and the simple sugars are subsequently isolated as hydrazones or osazones in the usual manner.

The present paper describes the properties of carbobenzoxy glucosamine and gives an example for the separation of a mixture containing glucosamine, mannose, galactose, and glucose into the individual components. The separation of glucosamine and glycine also is described. The application of this method to material of natural origin will be reported later.

EXPERIMENTAL

Carbobenzoxy Glucosamine—To a solution of 5.38 gm. of glucosamine hydrochloride (0.025 mole) in 25 cc. of water, 5.25 gm. of sodium bicarbonate were added, followed by 6.2 gm. of carbobenzoxy chloride in five portions, with shaking, during the course of an hour. After a few minutes clusters of fine needles started to separate. The mixture was kept in the refrigerator for a few hours, 25 cc. of chloroform were added to the white paste formed, and the precipitate was filtered off, washed with chloroform, and dried. It weighed 7.73 gm. (99 per cent of the theoretical yield). On recrystallization from 30 per cent methyl alcohol white lancet-shaped needles were obtained which melted with decomposition at 214° (corrected). The substance is dextrorotatory and shows

marked mutarotation with a final $[\alpha]_D^{24} = +75.4°$ (in pyridine). It reduces Fehling's solution and gives a negative ninhydrin reaction. It is soluble in glacial acetic acid and pyridine, in hot alcohol, and hot acetone, very slightly soluble in hot water; insoluble in benzene, chloroform, ethyl acetate. At 25°, 0.57 gm. of the compound is soluble in 100 cc. of water.

Analysis—$C_{14}H_{19}O_7N$.   Calculated.   C 53.6, H 6.1, N 4.5
313.1   Found.   " 53.7, " 6.2, " 4.5

Rotation—A 3.42 per cent solution in dry pyridine gave $[\alpha]_D^{24}$, initial = $+62.8°$; after 18 hours = $+72.4°$; after 24 hours = $+75.4°$. The unchanged compound could be recovered from the pyridine solution.

Carbobenzoxy glucosamine on treatment with palladium and hydrogen (7) gives glucosamine in almost quantitative yield, as shown in the following experiment. From 1.3 gm. of glucosamine hydrochloride dissolved in 5 cc. of water, the carbobenzoxy derivative was prepared as above. The crude product was suspended in 35 cc. of 2 N hydrochloric acid and decomposed with hydrogen in the presence of the palladium catalyst for 6 hours. By warming the reaction mixture to 40° the decomposition is speeded up. The filtered solution was concentrated *in vacuo* and the residue crystallized from water containing a little alcohol. In the first crystallization 0.67 gm. of pure *glucosamine hydrochloride* and from the mother liquors 0.54 gm. of the same material were obtained. The total yield of 1.21 gm. corresponds to 93 per cent of the glucosamine hydrochloride used.

Analysis—$C_6H_{13}O_5N \cdot HCl$.   Calculated.   C 33.4, H 6.6, N 6.5
215.6   Found.   " 33.4, " 6.7, " 6.2

Rotation—1.4 per cent aqueous solution; $[\alpha]_D^{25}$ (initial) = $+89.7°$; $[\alpha]_D^{23}$ (at equilibrium) = $+71.0°$.

Separation of Mixture of Glucosamine and Simple Sugars. Preliminary Experiments—Before using carbobenzoxy glucosamine for the isolation of glucosamine it was necessary to ascertain that simple sugars were not attacked by carbobenzoxy chloride. This was done in two sets of experiments. First, experiments were undertaken on the behavior of sugars when treated with carbobenzoxy chloride under the conditions outlined above. The fol-

lowing substances were examined: *l*-arabinose, *d*-ribose, *d*-xylose, *d*-glucose, *d*-mannose, *d*-galactose, *d*-fructose, glucuronogalactose from gum arabic (8).[1] With none of these compounds was an insoluble reaction product obtained.

It then had to be determined whether carbohydrates could be recovered unchanged after treatment with carbobenzoxy chloride. A solution of 1.0 gm. of *d*-mannose in 10 cc. of water was shaken with 1.9 gm. of carbobenzoxy chloride in the presence of 0.95 gm. of sodium bicarbonate for 1 hour. The mixture was extracted with chloroform in order to remove the carbobenzoxy chloride. On addition of phenylhydrazine to the aqueous solution which had been slightly acidified with acetic acid, 1.35 gm. of almost pure mannose phenylhydrazone were obtained (corresponding to 0.90 gm. of mannose) which, recrystallized from 60 per cent alcohol, melted at 201° (corrected). The melting point was not depressed on the admixture of an authentic sample of mannose phenylhydrazone. This experiment showed that mannose was not attacked by carbobenzoxy chloride under the conditions used.

Experiments were also carried out to show whether a large excess of sugars had a masking effect on the precipitation of carbobenzoxy glucosamine. This does not seem to be the case. For instance, from a mixture of 400 mg. of glucose and 20 mg. of glucosamine hydrochloride in 1 cc. of water, 22 mg. of the carbobenzoxy compound could be isolated.

Separation—A mixture of 2.0 gm. of *d*-galactose, 2.0 gm. of *d*-glucose, 1.0 gm. of *d*-mannose, and 1.3 gm. of glucosamine hydrochloride was dissolved in 10 cc. of water; 1.5 gm. of sodium bicarbonate and 2.0 gm. of carbobenzoxy chloride (in two portions) were added, and the mixture was shaken for 40 minutes. After chilling in the refrigerator, the precipitate which had formed was centrifuged off and thoroughly washed with ice water. It then was suspended in 45 cc. of 2 N hydrochloric acid, treated with hydrogen in the presence of the palladium catalyst for 6 hours, and the *glucosamine hydrochloride* was isolated as described above. The first crop of crystals weighed 0.59 gm. From the mother liquors 0.45 gm. was obtained; yield, 80 per cent of the glucosamine hydrochloride present.

[1] We are indebted to Dr. M. Heidelberger for a sample of this compound.

Analysis—$C_6H_{14}O_6N \cdot HCl$ (215.6). Calculated, N 6.5; found, N 6.3

Rotation—1.5 per cent aqueous solution, $[\alpha]_D^{25}$, at equilibrium = $+71.2°$.

The aqueous solution containing the sugars was acidified with acetic acid, extracted with chloroform, and diluted to a volume of 30 cc. On addition of 2 gm. of freshly distilled phenylhydrazine in 1 cc. of alcohol, 1.2 gm. of *mannose phenylhydrazone* (corresponding to 0.83 gm. of mannose) crystallized, which after recrystallization from 60 per cent alcohol melted with decomposition at 200° (corrected).

Analysis—$C_{12}H_{18}O_5N_2$ (270.1). Calculated, N 10.4; found, N 10.4

The filtrate from the phenylhydrazone was treated with benzaldehyde in the usual manner (9), concentrated, and divided into two equal portions. From one portion *d-galactose-α-methylphenylhydrazone* (0.61 gm.) was prepared, which after two recrystallizations from 30 per cent alcohol was obtained in the form of shining plates melting with decomposition at 194° (corrected).

Analysis—$C_{13}H_{20}O_5N_2$ (284.2). Calculated, N 9.8; found, N 9.5

From the other portion of the sugar solution *d-galactose-o-tolylhydrazone* (0.40 gm.) was prepared; it separated in long needles (from 96 per cent alcohol) melting with decomposition at 180° (corrected).

Analysis—$C_{13}H_{20}O_5N_2$ (284.2). Calculated, N 9.8; found, N 9.6

The combined filtrates from the galactose hydrazones were treated with formaldehyde according to the method of Ruff and Ollendorff (10). *Glucose phenylosazone* was prepared in the usual manner. After repeated extraction of the crude osazone with hot acetone 0.66 gm. of yellow needles were obtained, which after two crystallizations from 90 per cent alcohol melted at 206° (corrected).

Separation of Glucosamine and Glycine—A solution of 1.0 gm. of glucosamine hydrochloride and 1.0 gm. of glycine in 10 cc. of water was exactly neutralized with N sodium hydroxide, and 1.8 gm. of sodium bicarbonate and 3.5 gm. of carbobenzoxy chloride (in three portions) were added. Because of foaming the mixture

was diluted with water to 50 cc., shaken for ½ hour, and chilled in the refrigerator. Almost pure *carbobenzoxy glucosamine* had precipitated (0.884 gm.), melting at 211–213° (corrected). The filtrate was acidified with hydrochloric acid, when 1.93 gm. of *carbobenzoxy glycine* (7) precipitated, melting at 117–119°. The acid filtrate was concentrated at room temperature and made slightly alkaline. A second crop of carbobenzoxy glucosamine was thereby obtained, weighing 0.248 gm. The yields obtained correspond to 78 per cent of the glucosamine hydrochloride and to 70 per cent of the glycine taken.

The microanalyses reported in this paper were carried out by Mr. W. Saschek.

SUMMARY

The preparation and properties of carbobenzoxy glucosamine are described. By means of this derivative glucosamine can be separated from mixtures containing simple sugars and amino acids.

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LACTATE AND PYRUVATE IN BLOOD AND URINE AFTER EXERCISE

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The changing lactate content of body fluids is a classical problem in the physiology of muscular exercise (1). Interpretation of results from whole animals has usually been in terms of what is known about glycolysis in isolated tissues and in enzyme preparations. The analysis of anaerobic glycolysis in muscle has proceeded a long way since 1932 (22, 23), and the current schemes suggest that, if the source of the excess lactate found in the body after muscular exercise is the musculature, the concentrations of lactate and various other substances in the body after exercise should vary together. In the opinion of most workers pyruvate is the immediate precursor of lactate in muscle glycolysis. We have, therefore, estimated lactate and pyruvate in the blood and urine after severe exercise.

Our three subjects were healthy young men in moderately good athletic training. We drew a sample of the subject's blood from an antecubital vein without stasis, and took a sample of urine, before he ran. After he had run to exhaustion on a motor-driven treadmill at an 8.6 per cent grade, he lay quietly on a bed. Samples of blood were drawn at the stated intervals from an antecubital vein, and the subject urinated at the times noted. The first subject ran at 311 meters per minute for 55 seconds, the second at 233 meters per minute for 80 seconds, and the third at 311 meters per minute for 56 seconds.

Lactate analyses were made by the method of Friedemann, Cotonio, and Shaffer (11). The urine was prepared for analysis by treatment with copper-lime. The blood filtrates were not, because we have confirmed the statement of Cook and Hurst (5) that its use is unnecessary with tungstate filtrates from blood.

We used Peters and Thompson's (24) modification of the Neuberg-Case method for pyruvate estimations. This is a colorimetric estimation of the pyruvate as the 2,4-dinitrophenylhydrazone.

Table I gives the results in two experiments and Fig. 1 those in a third. All three experiments showed the same features. The lactate and pyruvate recovery curves for blood had similar

TABLE I

Lactate and Pyruvate in Blood and Urine

The values for blood are measured in mM per liter; the values for urine are the total output for stated periods in mM.

Blood				Urine			
Time	Lactate	Pyruvate	$\frac{\text{Lactate}}{\text{Pyruvate}}$	Period	Lactate	Pyruvate	$\frac{\text{Lactate}}{\text{Pyruvate}}$
Experiment I							
Before run	2.2	0.18	12	Before run, 60 min.	0.1	0.006	17
After " 5 min.	12.1	0.33	37	After run			
16 "	11.7	0.60	20	0–22 min.	6.1	0.01	610
26 "	9.1	0.41	22	0–50 "	7.7	0.04	193
40 "	5.1	0.27	19				
Experiment II							
Before run	0.8	0.11	7	Before run, 70 min.	0.1	0.02	5
After " 5 min.	7.3	0.29	25	After run			
15 "	7.3	0.36	20	0–10 min.	0.9	0.04	23
25 "	5.2	0.26	20	0–45 "	7.0	0.14	50
41 "		0.22		0–90 "	7.6	0.18	42
55 "	2.6						

shapes, though pyruvate was present in much smaller amounts than lactate. The ratio of mM of lactate to mM of pyruvate ranged from 7 to 44. In our three subjects the pyruvate maximum came later than the lactate, but both curves fell off in the same way. The shapes of the curves for urine were remarkably similar. Excretion of both lactate and pyruvate was complete in about 40 minutes. Here again the amount of pyruvate in relation to lactate was very small.

Spiro (31) showed that lactate is excreted in the urine after exercise. This has been confirmed many times (3, 4, 8, 9, 12, 13, 16–18, 27, 28, 30). Liljestrand and Wilson (17) have made the most complete study of urinary lactate excretion. They obtained samples of urine every 10 minutes from their subjects and constructed a complete curve for rates of excretion during recovery. They were also the first actually to isolate the lactate from urine after exercise, and to show that it is l-(+)-lactate. Jervell (13) studied lactate excretion in relation to its blood concentration. We confirm the conclusions of these earlier workers, that excretion
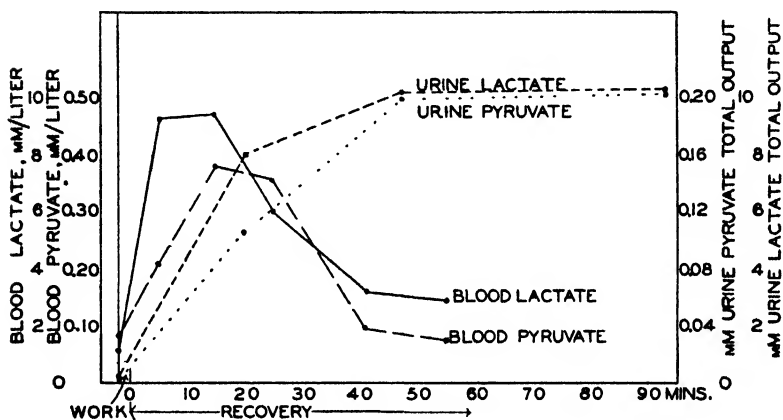
Fig. 1. The relation between lactate and pyruvate in blood and urine after hard running.

of lactate is complete about 40 minutes after work stops, and that increased blood lactate is followed by increased lactate excretion.

There was some doubt that the substance we estimated was actually pyruvate. Acetic acid 2,4-dinitrophenylhydrazide (6), and the 2,4-dinitrophenylhydrazones of such acids as oxaloacetic, glyoxylic, and mesoxalic would, if present, increase the value for pyruvate. Therefore, we prepared pyruvic acid 2,4-dinitrophenylhydrazone from 1 liter of urine and 400 cc. of blood collected from four subjects after they had run to exhaustion. We used the technique that Johnson (14) used for pigeon blood. It worked well for the blood, but we had to purify the urine hydra-

zone further by solution in toluene, precipitation from toluene with petroleum ether, and recrystallization from ethyl acetate with petroleum ether (Table II).

The yields suggest that most of the substance estimated by the Neuberg-Case method under our conditions is in fact pyruvic acid or some unstable precursor.

Fricke (10) failed to isolate pyruvate from the blood and urine of normal and diabetic humans, and Simon and Aubel (29) could not isolate it from dog blood.   Berthelot and Amoureux (2) reported its presence in the human small intestine.   Mendel, Bauch, and Strelitz (21) used a very indirect method to demon-

TABLE II

Properties of Pyruvic Acid 2,4-Dinitrophenylhydrazone Prepared from Various Sources

Source	Amount	M.p., corrected	Mixed m.p., corrected	Nitrogen*
	mg.	*°C.*	*°C.*	*per cent*
Urine................................	30	216	216	20.80
Blood................................	11	215–216	216	20.48
Synthetic..........................		216		20.55
Theory..............................				20.88

* The analyses were made by Mrs. Wellwood of the Converse Laboratory, Cambridge.   It is sometimes difficult to get off the last traces of nitrogen from 2,4-dinitrophenylhydrazones by the micro-Dumas method.   We have repeatedly analyzed this sample of synthetic hydrazone ourselves with theoretical results.

strate it in the serum of cancerous humans.   It has been isolated as the 2,4-dinitrophenylhydrazone from the urine of compensated diabetics (25), from pig serum (32), from the blood of pigeons deficient in vitamin B_1 (14), and from the urine of humans with beriberi (26).   We (15) have reported it in the urine of normal humans after exercise.

If we assume for both lactate and pyruvate the conditions of diffusion that Margaria, Edwards, and Dill (20) described for lactate, then the total excretion of excess lactate and pyruvate accounts for about 2 per cent of the total excess that the body has to dissipate after hard exercise for a short time.

We looked for but did not find methylglyoxal 2,4-dinitrophenyl-

osazone in any of our estimations.　Either methylglyoxal is formed during exercise in the tissues but does not reach the blood stream, or else it is not formed at all.　We interpret our data as support for the Embden-Meyerhof scheme for muscle glycolysis, although we realize that the excess pyruvate might be an oxidation product of excess lactate by the liver or some other tissue (19), or might be associated with the rise in blood sugar which follows hard exercise (7).　The curves are, however, consistent with the hypotheses that during or immediately after hard exercise, glycolysis takes place in the muscles according to the Embden-Meyerhof scheme; that lactate and at least one of its precursors, pyruvate, are formed in such excess that they appear in amounts greater than the resting value in the blood stream; and that they are removed in similar ways.

SUMMARY

1. The lactate and pyruvate recovery curves for blood and urine in young men after hard running are similar in shape, but in any sample of blood or urine, pyruvate is present in much smaller amounts than lactate.

2. Pyruvic acid from blood and urine collected after running has been isolated as the 2,4-dinitrophenylhydrazone.

3. These facts seem to support the validity of the Embden-Meyerhof scheme for muscle glycolysis *in vivo*.

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SOME WAX-LIKE CONSTITUENTS FROM EXPRESSED OIL FROM THE PEEL OF FLORIDA GRAPEFRUIT, CITRUS GRANDIS*

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Nelson and Mottern (1) examined the volatile constituents of grapefruit oil obtained by cold pressing the peel of Florida Common and Duncan type grapefruit, *Citrus grandis*, L., Osbeck.[1] The oil was distilled under reduced pressure to remove the bulk of the limonene fraction and then distilled with steam to remove the volatile constituents. The non-volatile residue remaining after distillation was of a wax-like consistency and represented 7.5 per cent of the original oil. The results of an examination of this fraction, which are reported in the present paper, would seem to indicate that the non-terpenoid constituents were derived from the cuticle wax through solution in the oil during the expressing process.

EXPERIMENTAL

The residue obtained on distillation of the grapefruit peel oil was a yellowish green, somewhat sticky wax at ordinary temperatures, while at 45–50° it melted to a brownish, viscous oil. Extraction with petroleum ether yielded approximately 62 per cent of soluble material together with a gummy residue of polymerized and oxidized terpene bodies which was discarded. The residue obtained on evaporation of the petroleum ether had an iodine number (Hanus) of 127.8 and a neutralization value of 14.3. A

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* Food Research Division Contribution No. 312.

[1] Nelson and Mottern neglected to mention the botanical name of the grapefruit in their paper on the constituents of the oil, and subsequently the work was described (2) under the heading *Citrus decumana*, a synonym of *Citrus grandis*.

portion (154 gm.) of this material was saponified with potassium hydroxide in benzene-alcohol (1:10) solution and the unsaponifiable material separated from the aqueous solution of soluble soaps by extraction with ether. The soap solution was acidified and extracted with ether to remove the acidic constituents. The unsaponifiable fraction amounted to 65 gm., equivalent to 42.2 per cent of the petroleum ether-soluble portion or 26.1 per cent of the original wax; whereas the acid fraction weighed 69 gm., equivalent to 44.8 per cent of the petroleum ether-soluble portion or 27.7 per cent of the original wax. The aqueous filtrates and washings were examined for glycerol but, owing to the presence of interfering oily products, its presence could not be demonstrated with certainty.

Solid Fatty Acids—The crude acids (69 gm.) were separated into liquid and solid fractions by the lead salt-ether method. The acids (4 gm.) derived from the insoluble lead salts were fractionally crystallized from acetone-alcohol solution and then from ethyl acetate. The main fraction melted at 84.0–84.5° and the combined lower fractions (0.5 gm.) melted indefinitely at about 60°. The higher melting fraction was converted into the corresponding ethyl ester and repeatedly crystallized from ethyl acetate. The ester melted at 69.2° and gave on analysis C 80.43, H 13.45. Calculated for $C_{34}H_{68}O_2$, C 80.23, H 13.48. With Cu Kα radiation and β Al_2O_3 as a reference the observed x-ray spacing[2] was 45.30 $\pm$ 0.30 Å.

The acid recovered from the ester after saponification melted at 87.7–87.9° and gave a spacing value of 78.60 $\pm$ 0.30 Å. The molecular weight by titration was found to be 477.8 and analysis gave C 79.76, H 13.11. Calculated for $C_{32}H_{64}O_2$, mol. wt. 480.5, C 79.92, H 13.42.

The molecular weight of the acid and the analytical values for the acid and the ester agree with the calculated values for dotriacontanoic acid, $C_{32}H_{64}O_2$, but the enhanced spacing of the acid and especially the ester, as well as the depressed melting points, indicate definitely that the acid is a mixture. Comparison of the values found for the acid from grapefruit with similar values

[2] Measurements of crystal spacings reported throughout this paper were kindly made by Dr. Sterling B. Hendricks, Fertilizer Investigations, Bureau of Chemistry and Soils.

recorded by Piper, Chibnall, and Williams (3) for synthetic acids and their mixtures indicates that the grapefruit acid is in all probability a ternary mixture of mean molecular weight corresponding to $C_{32}H_{64}O_2$.

Liquid Fatty Acids—Normally the ether-soluble product recovered in the lead salt-ether separation of the solid and liquid acids derived from plant cuticle waxes is found to consist of various mixtures of mono-, di-, and triethenoid acids. After conversion into the corresponding bromo derivatives the separation and identification of the constituents of these mixtures usually present no particular difficulty. However, in the case of the grapefruit wax difficulty was encountered owing to the fact, as will be developed later, that the fraction contained besides C_{18}-unsaturated acids, considerable amounts of terpene and terpene alteration products.

Linolenic Acid—The crude liquid acid fraction (57 gm.) was dissolved in ether, cooled to $-10°$, and brominated according to the method described by Lewkowitsch (4). Crystalline bromides which began separating during the bromination were collected on a filter, washed with ether, and recrystallized from benzene. The product (7 gm.) melted at 181–182° and gave on analysis C 29.72, H 3.26, Br 60.09. Since analysis indicated the possible presence of some tetrabromostearic acid, the product was repeatedly extracted with boiling ether, but no change in analysis or melting point was effected by this treatment. Purification was finally accomplished by fractional crystallization from alcohol-benzene solution whereby a quantity of unidentified solid (m. p. 188–189°) was separated from the hexabromolinolenic acid. Analysis of the purified product gave C 28.46, H 3.93, Br 63.15. Hexabromolinolenic acid $C_{18}H_{30}O_2Br_6$, requires C 28.51, H 3.99, Br 63.28.

Linoleic Acid—The ether solution after the removal of the hexabromides was freed of excess bromine and evaporated *in vacuo*. The residue was extracted with boiling petroleum ether and the solution set aside to permit the separation of the tetrabromides. The crystalline solids (10.5 gm.) which separated were collected by filtration and twice crystallized from petroleum ether containing a small amount of ether. The bromo derivative was obtained in the form of rosettes of needles melting at 113.5–114.5° and gave on analysis C 35.98, H 5.52, Br 53.46. Tetra-

bromostearic acid, $C_{18}H_{32}O_2Br_4$, requires C 36.00, H 5.38, Br 53.28.

Oleic Acid—After removal of the tetrabromides the petroleum ether filtrate was evaporated and the residue (43.5 gm.) debrominated with zinc dust in alcohol solution. Examination of the product recovered after debromination indicated that it consisted of a mixture of oleic acid together with a considerable amount of polymerized terpenoid bodies. The iodine number (Hanus) was abnormally low (18.1 compared to 90 for oleic acid) and dihydroxystearic acid could not be isolated from the mixture after oxidation with potassium permanganate.

Since it was not feasible to separate oleic acid from the debrominated mixture, a quantity (14.5 gm.) of crude mixed acids was methylated with 5 per cent hydrochloric acid in methanol. After removal of the bulk of the solid esters by chilling and filtering from a methanol solution, the liquid portion (12 gm.) was transferred to a molecular still (5) and fractionally distilled at a pressure of 0.3 μ. During the period of degassing and the early stages of distillation 5 gm. (approximately 40 per cent) of the charge collected in the liquid air trap. It consisted of a red oil of characteristic terpenic odor. Four ester fractions, representing 6.5 gm. or approximately 54 per cent of the still charge were collected between 80–95°. The first fraction, iodine number 93.5, consisted of nearly pure methyl oleate, iodine number 85.7. Fraction 2 (iodine number 111.6) consisted of methyl oleate together with an appreciable quantity of methyl linoleate. Fractions 3 and 4 (iodine numbers 143.5 and 148.6) consisted principally of methyl linoleate, iodine number 172.5. About 0.5 gm. of material remained in the still after the removal of the fractions mentioned above, but further distillation proved to be impracticable.

Unsaponifiable Fraction—The yellowish red, semisolid material representing the unsaponifiable fraction (65 gm.) was dissolved in hot alcohol and the solids which separated on cooling were removed by filtration. The filtrate was concentrated and the solid fraction separated in the same manner. By repetition of the process there were collected altogether 14.5 gm. of solids and a residue consisting of a red aromatic oil. The solid portion was fractionally crystallized from alcohol, whereby three fractions were obtained as follows: Fraction A, 1.5 gm. of white crystalline

material, difficultly soluble in alcohol, and melting at 240–245°; Fraction B, 2 gm. of amorphous solid, melting at 60–65°; Fraction C, 11 gm. of amorphous solid, melting at about 115°.

Ketone, $C_{30}H_{52}CO$—The material representing Fraction A was repeatedly crystallized from amyl alcohol-amyl acetate solution, from which it was finally obtained in the form of long, satiny needles, melting at 253–254°, and having a specific rotation, $[\alpha]_D^{20} = -20.1°$ and $-21.3°$. The results of combustion indicated the presence of 1 oxygen atom, but tests for the presence of hydroxyl and alkoxyl were negative. The compound, however, gave a positive test for the presence of a carbonyl group and it was therefore assumed to be a ketone. A portion of the compound was suspended in absolute alcohol, an excess of hydroxylamine hydrochloride and sodium hydroxide added, and the mixture refluxed for several hours. After recrystallization from amyl acetate, the recovered reaction product melted at 281–282°. The ketoxime after drying at 110° gave on analysis C 81.67, H 11.56, N (Dumas) 3.43. Calculated for $C_{30}H_{52}C:NOH$, C 81.68, H 11.73, N 3.08. Analysis of the original ketone gave C 84.48, 84.41, H 11.91, 11.90. Calculated for $C_{30}H_{52}CO$, C 84.47, H 11.90.

A C_{30} skeleton and a melting point above 250° indicate that the ketone is a member of the class of polyterpenoids which are referred to as sapogenins. The most commonly occurring fruit coat sapogenins are acids, principally ursolic and oleanolic, rather than ketones. This is the first instance in which we have encountered a ketonic sapogenin.

Hydrocarbons, $C_{29}H_{60}$ and $C_{31}H_{64}$—The material representing Fraction B (m. p. about 60°) was treated with concentrated sulfuric acid at 110–120° until addition of fresh acid to the recovered product no longer resulted in discoloration. The oxygen-free product was then fractionated from petroleum ether-acetone solution, whereupon there were obtained three main fractions and five additional ones derived from mother liquors.

The melting points, setting points, crystal spacings, and in some cases carbon and hydrogen values for the various fractions are recorded in Table I, together with comparative data for some pure synthetic hydrocarbons and their mixtures recorded by Piper, Chibnall, and coworkers (6). The transition points, which are not

recorded, were either considerably depressed (3–4° for the best fractions) or were too indefinite to be valuable as characterizing criteria.

The complex nature of the hydrocarbon mixture derived from the grapefruit is evident on comparison of the values for the various fractions of the naturally occurring substances with those for pure synthetic hydrocarbons and their mixtures. The principal components of the grapefruit hydrocarbon fraction appear to be n-nonacosane, $C_{29}H_{60}$, and n-hentriacontane, $C_{31}H_{64}$, together with variable amounts of lower homologues, and possibly some higher member.

TABLE I

Analysis, Thermal, and x-Ray Values for Grapefruit and Synthetic Hydrocarbons

Source	Melting point	Setting point	Spacing	C	H
	°C.	°C.	Å.	per cent	per cent
Grapefruit, Fraction 1..........	67.8–68.2	67.5–67.3	42.84	85.29	14.73
Synthetic $C_{31}H_{64}$...............	67.6–67.8	67.3	41.55	85.22	14.78
Grapefruit, Fraction 2..........	65.1–65.5	64.7–64.5	40.70	85.40	14.92
Synthetic $C_{29}+C_{31}$ (60:40)......	64 8–65.0	64.4	40.9	85.20	14.80
Grapefruit, Fraction 3..........	63.9–64.4	63.2–62.7	40.32	85.05	14.98
Synthetic $C_{29}+C_{31}$ (85:15)	64.0–64.2	63.5	39.7	85.19	14.81
Grapefruit, Fraction M1–1......	62.7–63.0	62.3–62.0	39.0	85.27	14.85
" " M1–2......	57.3–57.7	56.9–56.7	37	85.04	14.78
" " M1–3.......	55.7–55.9	55.5–55.2	36.9		
. " " M1–4.....	55.5–55.7	55.2–55.0	35.9		
" " M1–5.... .	53.9–54.2	53.3–53.1	35.4		

Phytosterol, $C_{28}H_{47}OH$—Since the material representing Fraction C gave an intense Liebermann-Burchard sterol reaction, it was heated under a reflux for 10 hours with an excess of phthalic anhydride and pyridine. The reflux mixture was poured into water and the pyridine and excess phthalic anhydride removed. The insoluble portion was separated, dissolved in ether, and the ether solution shaken with dilute sodium carbonate. The ethereal solution, which contained the sodium salts of the secondary alcohol phthalates, was washed with water, and the ether removed by evaporation. The residue was dissolved in a small amount of alcohol (75 ml.) and allowed to stand. Hydrocarbons and

ketones, which would have separated at this stage if present, were not detected; therefore the phthalyl ester was saponified and the regenerated product crystallized from dilute alcohol from which it was obtained in the form of plates melting at 132–133°.

A portion (0.9 gm.) of this material was acetylated with an excess of acetic anhydride and the acetyl derivative crystallized from 95 per cent alcohol. The main fraction melted at 112.5–113.5° and was unchanged by further crystallizations. Analysis gave C 81.42, H 11.37. Calculated for $C_{28}H_{47}O \cdot COCH_3$, C 81.38, H 11.39.

One portion (0.37 gm.) of the acetylated product was dissolved in ether and brominated dropwise with a solution of bromine in glacial acetic acid (1:30). The ether was permitted to evaporate spontaneously and the crystalline bromide which separated was filtered off and washed with glacial acetic acid. An additional quantity of the bromo derivative was recovered from the acetic acid mother liquors. Both fractions were crystallized from alcohol-ethyl acetate solution, from which the bromoacetyl compound was obtained in the form of plates and rods melting at 115°. Analysis gave C 59.95, H 8.32, Br 26.55. Calculated for $C_{28}H_{47}Br_2O \cdot COCH_3$, C 59.78, H 8.37, Br 26.54.

Another portion of the acetylation product was saponified with sodium ethoxide and the regenerated parent substance crystallized from alcohol-ether solution. The product melted at 137.5–138.5° and had a specific rotation, $[\alpha]_D^{20} = -0.37°$, in chloroform solution. Analysis gave C 83.72, H 12.17. Calculated for $C_{28}H_{47}OH$, C 83.92, H 12.08.

The analysis and chemical and physical properties of the secondary alcohol isolated from the grapefruit wax correspond to a phytosterol, $C_{28}H_{47}OH$. The sterol, however, does not correspond in chemical and physical properties to any completely characterized plant sterol of this formula and it is therefore concluded that the grapefruit product is either a previously unidentified phytosterol or a mixture of the commonly occurring plant sterols.

Unsaponifiable Oily Fraction—The reddish colored, viscous oil (50 gm.) which remained after removal of the solid portion of the unsaponifiable fraction was transferred to a molecular still and distilled at 10^{-4} mm. pressure. Distillation began at about

125° and continued up to 205°, when heating was discontinued owing to the fact that the condensing surface of the still became completely coated with a deposit of crystalline material. Approximately 35 per cent of the oily fraction distilled under these conditions. The various fractions collected during the distillation were all quite similar in character and consisted of yellow oils, varying in density from 0.996 to 1.000. The specific rotation in chloroform solution varied from $+106°$ to $+123°$, whereas the refractive indices of the fractions varied from 1.518 to 1.519 and the oxygen content approximately 16 per cent. Although no chemical entity could be isolated from the oily distillates, it was obvious that they consisted of oxygenated terpene bodies.

Umbelliferone, $HO \cdot C_6H_3 \cdot CH : CHCO$—The colored crystalline

material representing the still-head condensate was removed by dissolving the sublimate in amyl acetate. The solvent was removed by evaporation and the residue taken up in acetone, given a char treatment, and the solid reprecipitated by the addition of petroleum ether. The precipitate was filtered off and recrystallized from hot water. The product separated in sheaf-like aggregates of thin prisms which melted at 232.0–232.2°. The mother liquor, as well as fresh aqueous solutions of the crystalline product, gave an intense blue fluorescence on the addition of alkalies. The compound was somewhat soluble in alcohol, acetone, and amyl acetate but was precipitated by the addition of petroleum ether. The above reactions are those given by umbelliferone (*p*-hydroxycoumarin) and its isomers. After several recrystallizations from water and drying at 110° analysis gave C 66.83, H 3.87. Calculated for umbelliferone, $C_9H_6O_3$, C 66.65, H 3.73.

Acetyl Umbelliferone, $CH_3CO \cdot OC_6H_3CH : CHCO$—A portion of

the umbelliferone was acetylated by refluxing with acetic anhydride. The acetylated product was recovered and recrystallized from dilute alcohol from which it separated in long thin needles melting at 142.0–142.2°. After drying at 110° analysis gave C 64.95, H 4.17. Calculated for acetyl umbelliferone, $C_{11}H_8O_4$, C 64.69, H 3.95.

Whether the umbelliferone was present in the original oil or was produced as a result of thermal reaction during the distillation is not known with certainty, but since no additional quantity could be isolated from the gummy residue remaining in the still, it may be assumed to have been formed at or near the temperature at which it distilled.

SUMMARY

The non-volatile, waxy residue remaining after distillation of Florida grapefruit peel oil has been examined and the following constituents identified: solid fatty acids of mean molecular weight corresponding to $C_{32}H_{64}O_2$; linolenic, linoleic, and oleic acids; a sapogenic ketone, $C_{30}H_{52}CO$; hydrocarbons, $C_{29}H_{60}$ and $C_{31}H_{64}$; a phytosterol, $C_{28}H_{47}OH$; and umbelliferone, $C_9H_6O_3$. The nature of the constituents comprising the non-volatile residue of grapefruit peel oil indicate that they have their origin in the cuticle wax of the fruit which is dissolved by the oil during the pressing process.

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A CONVENIENT METHOD OF DETERMINING SMALL AMOUNTS OF AMMONIA AND OTHER BASES BY THE USE OF BORIC ACID

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Stover and Sandin (1) proposed the use of boric acid as the acid to trap ammonia for Pregl's micro-Kjeldahl method. Meeker and Wagner (2) recommended the use of boric acid following Stover and Sandin's technique in the macro- as well as the micro-Kjeldahl procedure.

We have found three determinations in particular where titration of bases in boric acid solution is of advantage: the urea aeration method of Van Slyke and Cullen (3), the nitrogen determinations by the micro-Kjeldahl distillation (4), and the new electrometric total base method of Keys (5).

In the *total base* method, the basic ions sodium, potassium, calcium, magnesium, and ammonium are separated from the proteins of blood by dialysis through a cellophane membrane by the passage of an electric current. The positive pole is a layer of mercury which forms amalgams with the basic ions. Above this layer of mercury there are exactly 2.00 cc. of 0.02 N H_2SO_4 which liberates the bases from the amalgam. The excess acid is then titrated with standard alkali. At this point the procedure may be varied to make use of boric acid. Here, approximately 2.0 cc. of 2 per cent boric acid are measured out (into the cathode tube) instead of the standard acid. The basic ions are dialyzed and converted into the corresponding borates. The solution then is titrated back to the original pH of the boric acid solution with 0.02 N acid. (The titration back to the original pH of boric acid is most easily done by adding indicator to a control tube of 2 per cent boric acid and titrating back to that color.)

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In the urea determination (3) urea is converted into ammonium carbonate by urease.   One tablet of Squibb's urease is added to 1 cc. of serum or plasma and 5 cc. of water.   This mixture is incubated at 37° for 20 minutes.   (These urease tablets contain the required amount of phosphate buffers for the optimum activity of urease.)   The NH₃ is liberated by the addition of 5 cc. of saturated K_2CO_3 and immediately aerated according to Van Slyke and Cullen for 30 minutes, trapped in 15 cc. of boric acid, and titrated back to the original pH of the boric acid with 0.01 N H_2SO_4 or 0.01 N HCl.   For controls 1 cc. of standard solution is similarly treated, while in the recovery studies 1 cc. of standard is added to 1 cc. of serum and treated as described.

In the *micro-Kjeldahl* distillation the same principle may be used, boric acid being substituted for standard HCl or H_2SO_4. At this point, we would like to mention an innovation in the former nitrogen determination wherein the expensive and time-consuming Kjeldahl apparatus is eliminated.   The digestion is carried out with a digestion mixture of 0.25 gm. of K_2SO_4, 0.1 gm. of $CuSO_4$, and 1 cc. of concentrated H_2SO_4 for 0.1 cc. of serum.   A few particles of selenium metal may be used as a catalyst.   After the digestion of the nitrogenous materials to $(NH_4)_2SO_4$, instead of transferring to the Kjeldahl still, we found that transferring to the tubes of the Van Slyke-Cullen urea apparatus was less time-consuming, eliminated the use of the expensive Kjeldahl stills, and was just as accurate.   The transfer of the digested material is accomplished by three washings, totaling about 8 cc. of water.   The sample is treated then with 6 cc. of 40 per cent sodium hydroxide-thiosulfate reagent. The NH₃ is then aerated, instead of distilled as in the Kjeldahl method, trapped in 15 cc. of boric acid, and titrated as before. The aeration if vigorous is complete in 30 minutes but for safety's sake 45 minutes are recommended.   The aeration is similar to the urea aeration of Van Slyke and Cullen (3).   This modification is especially useful when a large number of samples are to be determined.

In connection with the ammonia titrations we have utilized a mixture of methyl red and methylene blue in the following proportions: 100 cc. of 0.03 per cent methyl red and 15 cc. of 0.1 per cent methylene blue.   Enough of this indicator is added to

boric acid to give a light purplish solution. If the color is too blue, add more methyl red. For total base estimation a 0.03 per cent solution of methyl red alone was found more satisfactory.

When the solution is acid, the red of the methyl red plus the blue of methylene blue results in a light purple solution. When the methyl red is above pH 4.3, the yellow of the methyl red plus the blue of methylene blue gives a *green* solution. The change from green to purple on addition of acid in titrating back to the original pH of boric acid is very sharp. When methyl red is used, there is a sharp change from yellowish brown to pink.

· These indicators may be mixed with the 2 per cent boric acid and stored in bottles, since there is very little change on standing.

TABLE I

Electrometric Estimation of Total Base with 0.2 Cc. Samples, by Boric Acid
The values are expressed in milli-equivalents.

Total base present......	Sample 1 0.0200	Sample 2 0.0300	Sample 3 0.0400
Total base found	0.0200	0.0302	0.0400
	0.0202	0.0302	0.0400
	0.0202	0.0300	0.0402
	0 0198	0.0298	0.0400

Results

Typical results for total base, urea, and Kjeldahl nitrogen estimations are given in Tables I, II, and III, respectively. The results show that the method with boric acid is capable of good accuracy. In the biochemical laboratory where large numbers of total protein, non-protein·nitrogen, albumin, urea, and total base determinations are required, the use of boric acid has been found to be convenient, time-saving, and reliable.

Preparation of Standard Solutions

Total Base—5.2605 gm. of c.p. sodium chloride and 1.3614 gm. of c.p. anhydrous monopotassium phosphate were dissolved in water and transferred quantitatively to a 1000 cc. volumetric flask and made up to mark. This solution contained 100 milliequivalents of base in 1000 cc. of solution. Another standard was made up similarly, with 8.1830 gm. of sodium chloride and

1.3614 gm. of monopotassium phosphate. This solution contained 150 milli-equivalents of base in 1000 cc. of solution.

Urea—2.144 gm. of recrystallized urea were dissolved and made up to mark with water in a 1000 cc. volumetric flask. 1 cc. of this solution contains 1 mg. of urea nitrogen.

TABLE II

Urea Nitrogen Estimations with Boric Acid to Receive Ammonia

In recovery studies, 1 cc. of urea standard was added. For controls 1 cc. of urea standard was used.

The values are expressed in mg.

Urea N	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Present in 1 cc. serum	1.070	0.135	0.175	0.196	2.520
Calculated	2.070	1.135	1.175	1.196	3.520
Found	2.060	1 127	1.175	1.190	3.516
Values found in standard solutions (1.000 mg. present)	1.000	0.999	0.990	0.999	0.998

TABLE III

Aeration of Standard Solutions of $(NH_4)_2SO_4$ with Boric Acid in Receiver

The values are expressed in mg.

N present..............	Sample 1 1.000	Sample 2 1.500	Sample 3 2.000
N found	0.989	1.498	1.998
	0.996	1.487	1.998
	1.010	1.509	1.991
	0 992	1.492	1.990

Nitrogen—4.7193 gm. of recrystallized ammonium sulfate were dissolved and made up to mark with water in a 1000 cc. volumetric flask. 1 cc. of this solution contains 1 mg. of nitrogen.

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HEPARIN: A MUCOITIN POLYSULFURIC ACID

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Extensive studies on heparin by Howell resulted in 1928 (1) in
the conclusion that it is of carbohydrate nature, containing a
hexuronic acid, probably glucuronic acid.   The purest samples
showed a very high content of ash and from the acid hydrolysate
crystals of calcium sulfate were obtained.   Charles and Scott have
through their work published in 1933 (2) greatly facilitated the
preparation of pure heparin in quantity and have shown that it is a
common tissue constituent.   Its carbohydrate nature was con-
firmed by them, as also by Fischer and Schmitz (3).   One of the
present authors, using the method of Charles and Scott for the
preparation of pure heparin, was able to show in 1935 (4, 5) that
protein-free heparin samples contain a hexuronic acid, as found by
Howell, a hexosamine, and a large amount of ester sulfates.   The
ratio between the two sugar components was 1:1.   The known
constituents together made up about 90 per cent of the heparin,
which therefore was considered to be a polysulfuric ester of chon-
droitin or some closely related substance.

In the meantime much evidence accumulated that one had
been dealing with pure heparin.   First of all the synthetic anti-
coagulants were found to be polysulfonic acids.   Furthermore,
one of us (6, 7) succeeded in activating ordinary polysaccharides
such as cellulose, starch, glycogen, pectic acid, chitin, and chon-
droitinsulfuric acid by introducing sulfate groups with chloro-
sulfonic acid in pyridine, a principle simultaneously applied suc-
cessfully by Chargaff, Bancroft, and Stanley-Brown (8).   In
cataphoretic experiments on heparin plasma Theorell, Eisler, and
Rosdahl (9) found that heparin migrated to the anode, causing
coagulation on the cathode side of the U-tube.   In spite of all

the indirect evidence available a complete elucidation of the chemical nature of heparin would be more convincing, and the following investigation was undertaken with that object.

Since our first report Lipmann and Fischer (10) and Schmitz (11) have claimed that their original preparations of liver heparin were free from sulfur. They give, however, no strong supporting evidence. Thus, Schmitz was surprised not to find any ash in his heparin, but, according to his description, he was working with the free acid, which is volatile on ignition. Only the neutral salts give a residue of sulfates. Fischer admits that a recently prepared heparin from the lungs contains 9.3 per cent sulfur and is identical in activity with our samples. Since we show below the chemical identity of lung heparin with heparin from liver, further discussion of a sulfur-free heparin appears futile.

Acid Hydrolysis of Heparin—On comparing the rate of hydrolysis of heparin with that of chondroitinsulfuric acid, we found a striking difference. On boiling with acids chondroitinsulfuric acid readily breaks down to chondrosine, which then resists further heating. On boiling in a water bath with 7.5 per cent (by volume) sulfuric acid, the reducing power expressed as glucose (Shaffer-Somogyi) after 5 minutes corresponds to 13 per cent and after 1 hour to 35.6 per cent of the organic substance. When heparin is hydrolyzed under the same conditions, the corresponding figures are 2.9 and 17.2 per cent.

The difference was at first believed to be due to differences in structure, possibly in the glycosidic linkages. When we found that the base of heparin was glucosamine and not galactosamine, it was clear that we were dealing with another substance.

On following the rate of hydrolysis of different heparin samples we discovered that the samples recovered from the easily soluble brucine salts showed a higher rate of hydrolysis, while those recovered from the insoluble brucine salts with a higher sulfur content and stronger heparin activity were more stable. This remarkable behavior was studied on the brucine fractions of two different samples of liver heparin and on the insoluble fraction of heparin obtained from the lungs. The figures found on hydrolyzing the different fractions of one sample of the liver heparin as recovered from their brucine salts are given in Table I. The other sample behaved similarly. Of the lung heparin only the less

soluble brucine fraction was hydrolyzed after removal of the brucine. Its sulfur content, rate of hydrolysis, and optical activity prove its identity with the heparin from the liver. Its effect on the time of coagulation was found to be in agreement herewith. Generally, however, heparin samples prepared from the lungs show a considerably lower activity, the amount of insoluble brucine salt obtained being comparatively small and rather difficult to isolate. Once separated, however, it has the properties of the corresponding heparin fraction from the liver.

TABLE I

Rate of Hydrolysis of Heparin Samples Compared with Their Degree of Esterification

Lung heparin	Liver heparin				Chondroitinsulfuric acid	Synthetic chondroitin polysulfuric acid
S calculated on basis of dry substance						
per cent	per cent	per cent	per cent	per cent	per cent	per cent
10.95	10.70	6.53	4.49	1.44		10.9
Hydrolysis at 100° in 7.5% (by volume) H_2SO_4						
min.						
5	1.89	1.26	3.76	4.65	7.55	13.0	7.4
10	2.83	2.52	5.30	7.76	12.6	20.5	15.4
20	5.11	2.99	11.7	14.2	17.7	31.4	24.5
40	8.65	7.08	18.9	23.3	25.3	35.0	27.8
60	11.80	10.22	23.2	28.7	30.5	35.6	29.8

The figures give the reducing power (Shaffer-Somogyi method) expressed as glucose in per cent of organic material.

As is evident from Table I, the more highly esterified heparin samples show greater resistance to acid. There is a direct proportionality between sulfur content and stability, which is most probably caused by the introduction of the sulfate groups.

The fractionation of the brucine salt of heparin was thus useful in another way. It had already helped toward an understanding of the sulfate linkage in heparin, when samples containing 2.5 atoms of S to each molecule of uronic acid and hexosamine could be divided into fractions corresponding to supposed chondroitindisulfuric and chondroitintrisulfuric acids. Secondly, the greater

heparin activity of the samples with the higher sulfur content was a clear indication as to the chemical nature of the active groups in heparin. The greater resistance of the stronger heparin preparations to acids may explain why the color reaction with naphthoresorcinol and hydrochloric acid is negative (2, 5) or only weakly positive (1), a source of confusion in the earlier discussion. Probably the necessity of applying a stronger acidity and a higher temperature in the Tollens-Lefèvre analysis than that recommended for ordinary glycosides containing hexuronic acid arises from the same cause.

This behavior was also demonstrated on chondroitinsulfuric acid itself after introducing three additional sulfate groups, though it was less evident here. A sample of the synthetic heparin containing 10.9 per cent sulfur prepared by Bergström (7) from chondroitinsulfuric acid by means of chlorosulfonic acid in pyridine was used for the hydrolysis (Table I).

Fractionation As Brucine Salt—As previously stated (5) the purified heparin preparations could be fractionated as brucine salts, the fractions obtained showing a different sulfur content and an activity varying with the degree of esterification. This finding very strongly indicated that heparin was a polysulfuric ester of chondroitin or some closely related substance. At that time only the insoluble brucine fraction with its greater heparin activity was more thoroughly studied. These experiments were accordingly repeated and the fractions analyzed. 7 gm. of a heparin preparation were electrodialyzed to neutral reaction of the cathode liquor. The activity of the material was 60 to 70 per cent of that of the standard preparation (*cf.* (5) Sample 2 of Tables I and II). After neutralization with brucine dissolved in methanol, the solution (700 ml.) was frozen and thawed repeatedly until clear, and then filtered. The mother liquor was concentrated, the brucine removed with alkali and chloroform, and the heparin precipitated with 1.5 volumes of acetone. In 24 hours a sticky mass had separated, which was dissolved in a little water and reprecipitated with 8 to 10 volumes of acetone. The yield of air-dried substance was 1.67 gm.

The less soluble brucine salt was dissolved in 700 ml. of boiling water, filtered through a hot funnel, and the filtrate cooled and frozen, and frozen again if it was not clear on thawing. This pro-

cedure was repeated three times with the precipitate. The insoluble brucine fraction had thus been redissolved in hot water four times. The amounts recovered and the composition of the different fractions are given in Table II.

The Tollens-Lefèvre analysis was performed as described earlier ((5) p. 1823). Analysis of a sample of chondroitinsulfuric acid and

TABLE II

Heparin Fractions Recovered from Brucine Salts

Substance recovered from	Air-dried substance	Per cent of dry substance			CO₂	Uronic acid	Hexosamine (Elson and Morgan method (12))		S per molecule uronic acid	Heparin activity
		Ash	S	N			Found	Calculated		
	gm.				*per cent*	*per cent*	*per cent*	*per cent*	*moles*	*per cent of standard*
Mother liquor										
1st	1.67	14.35	5.25	3.94	6.57	28.95	22.5 23.0	26.8	1.1:1	25– 50
2nd	0.400	27.3	8.99	3 05						50
3rd	0.210	31.6	9.83							
4th	0.100	30.9	9.93							
5th	0.050		11.33							
Insoluble brucine salt	1.464	34.0	12.03		5.88	25.90	21.3 22.8	23.9	2.8:1	120–130
Mother liquor										
1st	0.177	19.7	1.44							
2nd	0.360	27.2	4.48							
3rd	0.120	21.3	6.53							
4th to 8th	0.320	30.1	10.83							
Insoluble brucine salt	0.420	34.6	10.68							

the glucuronogalactose from gum arabic gave the following figures: for the former 6.48 per cent CO_2 (calculated according to the N content, 6.63) and for the latter 11.5 per cent (calculated, 11.22).

In a second experiment performed in a similar way the insoluble brucine salt was redissolved eight times. The first mother liquor containing the easily soluble brucine salt was concentrated to a

volume of 50 ml. before filtration.   On removal of the brucine a
fraction with a very low sulfur content, 1.44 per cent, was obtained.
The ash and sulfur contents of the fractions recovered after
removal of the brucine are also shown in Table II.

In a third experiment, in which the brucine salt was redissolved
in hot water four times, the brucine was removed by means of cal-
cium hydroxide, whereby calcium salts were obtained.   The salts
were very hygroscopic.   Analyses of the fractions are given in
Table III.

For comparison a sample of lung heparin was treated in the same
way.   As already stated, only a small amount of insoluble brucine
salt was obtained.   After removal of the brucine the sulfur content

TABLE III

*Influence of Degree of Esterification upon Heparin Activity and Optical
Rotation of Samples of Heparin Fractionated with Brucine*

Substance recovered from	Air-dry sub-stance	Mois-ture	Ash*	S*	N*	Ca*	$[\alpha]_D^{20}$*	Heparin activity
	gm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*degrees*	*per cent of standard*
1st mother liquor	0.57	20.0	22.7	3.44	5.11		+15	3
2nd " "	0.75	20.9	30.3	6.47	3.10		+43	40
3rd to 5th mother liquors	1.54	24.3	37.5	10.09	2.42	10.82	+41	55
Insoluble brucine salt	4.1	25.2	36.1	12.18	2.24	11.04	+66	135

* Calculated on the basis of the dry substance (110° *in vacuo*).

of this fraction was 10.95 per cent, calculated on the basis of dry
substance.   It hydrolyzed in the same way (see Table I) and
showed the same anticoagulating effect as the corresponding frac-
tion from the liver heparin. $[\alpha]_D^{20}$ was +52.2°.   Thus its rotatory
power also corresponded to the degree of esterification (10.95 per
cent S).   As is seen in Table III, $[\alpha]_D$ for liver heparin with a sulfur
content of 10.09 was +41°, and for another sample with 12.18
per cent S, +66°.   It is evident therefore that the heparin of the
lungs is identical with the product of the liver.   We also found
the hydrochloride of the amino sugar of lung heparin to crystallize
in tetragonal plates, as is the case with glucosamine hydrochloride.

Amino Sugar of Heparin—In heparin there is 1 molecule of

hexosamine for each molecule of hexuronic acid. This was determined on the earliest samples by the micromethod of Elson and Morgan (12) and confirmed on later samples. The amino sugar of heparin has recently been shown to be glucosamine (13). This sugar occurs regardless of the degree of esterification.

When the high heparin content of the lungs was considered, it was at first assumed that the heparin was a polysulfuric ester of the chondroitin of the cartilage. It is now evident that heparin belongs to the mucoitin group, so that general interest in the mucoitinsulfuric acid of Levene will of course be greatly stimulated.

Acetyl Content of Heparin—According to Fürth, Herrmann, and Schott (14) the amino sugars in the mucoproteins are monoacetylated, as also is chondroitinsulfuric acid. The same is the case with heparin. In the purest samples of heparin the amino group is not free. In the Van Slyke procedure no nitrogen is liberated. Attempts to determine the acetyl content, however, were not successful. Hydrolysis with alkaline methyl alcohol as recommended by Kuhn and Roth (15)-liberated a large excess of acid when applied to chondroitinsulfuric acid, as was also found by Friedrich and Sternberg (16). On the other hand hydrolysis with toluenesulfonic acid, either as recommended by Friedrich and Rapoport (17) or after neutralization to 50 per cent as recommended by Friedrich and Sternberg, does not liberate acetic acid from the purest heparin preparations. At first it appeared that the acetyl group had been removed through the influence of the alkali during the preparation of the substance, but when it was found that the amino group was not free and that the highly esterified polysaccharide was extremely resistant to acid hydrolysis, it was clear that the acetyl group could not be liberated in this way. On application of 10 per cent (by volume) sulfuric acid as used by Levene and after hydrolysis for 3 hours in a water bath, the acid liberated corresponded to about 50 per cent of the calculated acetyl content. The yield could not be augmented by increasing the temperature or the strength of the acid. Acetic acid was identified in the distillate as the silver salt.

Hexuronic Acid of Heparin—The hexuronic acid of heparin is most probably glucuronic acid. Howell has stated that there is no formation of mucic acid on oxidation with nitric acid. We have found the same. Our attempts to isolate saccharic acid were

Heparin

not successful. The resistance of heparin towards acids will probably make the isolation of the uronic acid difficult and costly.

The existence of the uronic acid is not to be doubted even if the naphthoresorcinol reaction is generally negative. We have on one occasion found a strongly positive reaction, but generally no violet color develops, probably because the stability of heparin prevents the liberation of the uronic acid in a few minutes. When more drastic hydrolysis is attempted, the uronic acid is destroyed. The content of hexuronic acid in the heparin samples is, however, of considerable importance, since conclusions can be drawn from it as to the purity of the samples.

The heparin samples of Table III were analyzed by the micromethod of Burckhart, Baur, and Link (18) with use of a water

TABLE IV

Uronic Acid Content of Heparin Samples of Different Degrees of Esterification
(See Table III)

Sample from	Sub-stance	CO₂		CO₂ cor-rected	Uronic acid
	mg.	*mg.*	*per cent of dry substance*	*per cent*	*per cent of dry substance*
2nd mother liquor (0.75 gm.)	125	7.075 7.05	7.14	6.68	29.45
3rd to 5th mother liquors (1.54 gm.)	150	6.50 6.59	5.77	5.4	23.80
Insoluble brucine salt (4.1 gm.)	150	6.31 6.25	5.6	5.24	23.10

cooler as recommended by Meyer and Palmer (19). 100 to 150 mg. of heparin were taken for each analysis, giving 6 to 7 mg. of CO_2. This was caught in ascarite in a Pregl tube. The increase in weight without the sample was 0.11 mg. on repeated tests; time of hydrolysis, 4 hours; bath temperature, 135°; concentration of hydrochloric acid, 20 per cent. When the method was applied to two samples of chondroitinsulfuric acid, the uronic acid figures were 7.0 and 5.8 per cent too high as calculated from the nitrogen content. While a lower concentration of the acid might have given theoretical figures, we preferred this technique because of the agreement between analyses of 1 to 2 per cent. On calculating the uronic acid content of heparin, therefore, we made a reduction of 6.5 per cent. The results are shown in Table IV.

To the content of uronic acid thus found was added 1 equivalent of hexosamine and acetic acid less 2 moles of water. If the ash (the first sample) or the content of calcium and sulfur with oxygen is added, the analyses account for 90.5, 84.8, and 88.8 per cent of the samples.

<center>DISCUSSION</center>

The heparin preparations described are polysulfuric esters of mucoitin. The analysis accounts for about 90 per cent of the preparations. The chemical nature ascribed fits in very closely with all that is known about heparin. It is quite resistant to the ordinary enzymes and to moderate temperatures. Basic lead acetate is the only metal salt to give a precipitate with heparin, except barium hydroxide. The first reagent also precipitates chondroitinsulfuric acid, but the second did so only after introduction of more sulfuric acid groups into the molecule. The insolubility of heparin in all states of purity in 50 per cent acetone is in agreement with this.

The direct evidence given of the purity of these preparations is strongly supported by the activation of ordinary polysaccharides by the introduction of sulfuric acid groups, although the synthetic products show far less heparin activity than the natural substance.

The information obtained upon comparing different heparin samples also points in the same direction. With an increasing sulfur content the activity increases, although a direct proportionality is not evident, as the preparations with low sulfur content show a lower activity than might be expected from their content of ester sulfates.

According to these findings, heparin is not a definite chemical compound, but a mucoitin polysulfuric ester. From the sulfur content it can be stated that at least a trisulfuric acid is present in it, mixed with di- and monosulfuric esters. The latter can be separated from the former as easily soluble brucine salts. If the mucoitin of heparin should prove to have the same structure as cellulose and starch, there would still be a possibility of obtaining a tetrasulfuric acid, as indicated at the positions marked x in the accompanying structural formula. This could possibly be separated from the others by means of cataphoresis.

HC⸺
|
HCOH x
|
x HOCH   O   O
|
HC⸺
|
HC⸺
|
COOH

HC⸺
|
HCN$^{H}_{Ac}$
|
x HOCH   O   O
|
HC⸺
|
HC⸺
|
H$_2$COH x
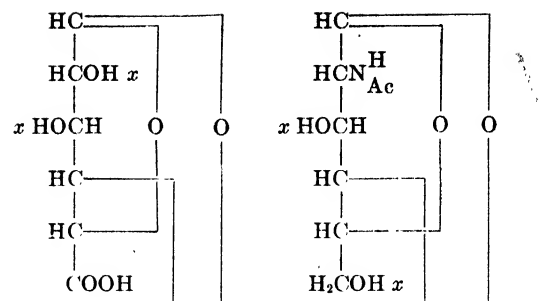
The recent statement made by Charles and Scott (20), that they have succeeded in crystallizing a heparin sample from the lungs, need not be in disagreement herewith. Their sample contained 11.5 per cent sulfur calculated on free acid. Since the calcium salt reported in Table III contains 12.18 per cent sulfur, which makes 13.6 per cent sulfur in the free acid, it is evident that our liver heparin represents a higher degree of esterification than the crystalline product from the lungs.

It is quite possible that certain chemical affinities of definite groups exert the anticoagulating effect, but as the facts stand it seems as if the activity of pure heparin must be due to its extraordinarily strong ionic charge in combination with a certain, not too small, molecular size.

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AMMONIA CONTENT, pH, AND CARBON DIOXIDE
TENSION IN THE INTESTINE OF DOGS*

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In the course of studies on the chemical changes in intestinal obstruction a striking change in the base content of the intestinal juice occurred. The concentration of fixed base decreased 5 to 27 per cent and the ammonia increased to a concentration ranging from 72 to 148 mg. per cent (1). Although Bliss (2) had reported ammonia in the vomitus of nephrectomized dogs, he seems not to have answered the criticism of Benedict and Nash (3) that it could have arisen from processes of digestion and putrefaction lower in the intestine. The extensive chemical analyses by de Beer, Johnston, and Wilson (4) of intestinal juice did not include ammonia. The decrease in fixed base of the juice, as mentioned above, suggested that the ammonia was produced to conserve base, as is the case in the kidney. Accordingly, this report is concerned with the effect of acidosis upon the ammonia content of the intestinal juice and the effect of various concentrations of ammonia upon the acid-base composition of the juice.

Procedure

Healthy dogs with Thiry-Vella fistulæ made in the first portion of the jejunum were used. Secretion was stimulated by inflating a rubber balloon in the fistulous bowel. Ordinarily, the juice was collected in a colostomy bag which was fastened to the dog's abdomen. When it was desired to collect the juice or the contents of the fistula without loss of CO_2, the dog was placed on its back and the juice was forced out by intestinal activity into a cup which was made by cutting off the open end of a 50 cc. syringe. The

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cup which would contain oil was sealed to the abdomen by collodion and held in place by a belt. This technique was used because in Thiry-Vella fistulæ the bacterial flora are fewer and the prompt escape of juice from the fistulæ minimizes changes in concentration due to absorption.

In most of the studies upon pH and CO_2 tension of the jejunum, because the rate of secretion was too slow to yield sufficient juice in many of the dogs, an isotonic NaCl-phosphate buffer of pH 7.0 was introduced into the upper end of the fistula at such a rate as to allow about 18 cc. of contents to escape into the cup within 2 hours. This procedure seemed justified in regard to pH determination, because the pH of the jejunal contents was practically the same as the pH of the succus entericus, although the total CO_2 and CO_2 tension of the latter were much higher. In these cases, the sample of succus entericus for ammonia analysis was collected either before or within 2 hours after the buffer had been passed through the intestine. The chemical methods used to obtain the data of Table I were those previously reported (1).

The CO_2 and pH determinations of Table II were made within 3 hours of collection of the samples, during which time the samples were kept in ice water. The CO_2 analyses were carried out by the Van Slyke manometric method (5) and the pH determined by a glass electrode.[1] The ammonia analyses were made by the aeration method, within 2 days of collection. It had been determined that within this period there was no increase in the ammonia content when toluene was added and the samples kept in the cold room. This was true even when urea was added to the succus entericus.

<div align="center">DISCUSSION</div>

In twenty-six experiments, acidosis was produced in ten dogs. The acidosis was produced in four experiments by exclusive fat feeding, in three experiments by fasting, and in nineteen experiments by $CaCl_2$ and HCl administration. In some cases the basal diet consisted of Purina Dog Chow and in others it was starch and butter. The effect of acidosis upon the chemical composition of intestinal juice and the corresponding blood serum is shown by representative data in Table I. In six of the twenty-six experi-

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[1] The Coleman pH electrometer was used.

ments the dogs were placed in metabolism cages for continuous collection of urine which was analyzed for urea and ammonia.

Acidosis, regardless of how it was experimentally produced, did not result in a permanent increase in the ammonia content of the juice. In four dogs there was an immediate decrease in the concentration of ammonia, in one there was no significant change, and in two there was an initial increase followed by a decrease. When the acidosis was maintained for several days and became quite severe, as in Dogs 5, 6, and 7 when they were fed the carbohydrate diet, the ammonia content rose above normal. This is believed to represent a destruction of tissues rather than a physiological reaction. The failure of the intestinal ammonia to increase simultaneously with urinary ammonia in the metabolism experiments lends support to this idea. In these experiments the total urinary ammonia increased on the average 2.1 to 9.3 times or the average ammonia to urea ratio increased 8.4 to 37 times, and yet the ammonia content of the succus entericus, if it increased at all, was less than 20 per cent and was paralleled by an increase in urinary urea.

In all cases the concentration of fixed base in the juice declined. The percentage decrease ranged from 6.1 to 32.0, with half of the values 14 per cent or more. In no case did there seem to be any reciprocal relationship between the concentrations of ammonia and fixed base, as one would expect if this ammonia reaction were to conserve base. In the case of Dog 5 in the first experiment, both fixed base and ammonia decreased, and in Dog 6 on the nonprotein diet, there seems to be a reciprocal relationship but the ammonia content is no greater than normal, whereas the fixed base has decreased 32 per cent. It apparently made no difference whether acidosis was produced on a diet such as Purina Dog Chow, which gave a higher juice ammonia (Dogs 5 and 6), or on a carbohydrate diet which resulted in a low ammonia content (Dogs 4 and 7). A reciprocal relationship between the concentrations of fixed base and ammonia in the juice was not demonstrated. Although normally the concentrations of fixed base in the juice and blood serum are about the same, in acidosis (exception, Dog 4) a greater reduction in concentration occurred in the juice. This was most marked in Dog 6 on the non-protein diet and in Dog 3.

The extensive studies of Gamble and his associates (6) of acidosis

produced experimentally in clinical cases by the ingestion of CaCl$_2$ or NH$_4$Cl demonstrated the following chemical changes: (1)

<div align="center">TABLE I</div>

Effect of Acidosis and Diet upon Concentration of Fixed Base and Ammonia in Intestinal Juice of Dogs

The data are expressed in milli-equivalents per liter.

Dog No.	Diet	Condition	Juice				Serum	
			Fixed base	NH$_2$	CO$_2$	Cl	CO$_2$ capacity	Base
1	Purina Dog Chow	Normal	150.5	10.5			21.4	163.1
	Fat	Acidosis	143.5	2.3			19.7	150.1
		"	140.5	4.1			19.5	158.2
2	Purina Dog Chow	Normal	149.4	11.6			20.6	155.1
	Fat	Acidosis	138.1	3.2			17.6	156.2
3	Purina Dog Chow	Normal	141.5	13.0			18.4	150.6
	Milk, Dog Chow + CaCl$_2$	Acidosis	119 5	10.0			14.8	149.0
4	Carbohydrate	Normal	163.8	3.1	17.0	126.7	20 0	164.2
	" + CaCl$_2$	Acidosis	153 0	3.3	15.5	101.4	15.1	149.0˙
5	Purina Dog Chow	Normal	156.0	13.2	22.0	126.7	20.1	159.5
	" " " +	Acidosis	152.9	21 0	12 5	121.1	10.0	159.5
	CaCl$_2$	"	142.1	8.8	12.1	121.1	13.2	158 8
	Carbohydrate + CaCl$_2$	"	133.5	10.9	8.0	115.5	11 2	162.5
	Fasting + CaCl$_2$	"	135 2	17.3	15.7	112.6	15.6	150.6
6	Purina Dog Chow	Normal	161.0	18.0	22 5	129.5	18.2	155.6
	" " " +	Acidosis	142.1	16.0	12.9	112.6	9 3	159.6
	CaCl$_2$	"	113.0	8 0	7.3	102.2		
	Carbohydrate + CaCl$_2$	"	109.0	18.0	14 0	95.7	10.4	151.3
7	"	Normal	150.5	4.1	17.7	121.1	19.3	156.7
	" + CaCl$_2$	Acidosis·	145.0	23.5	10.7	149.3	9.8	154.7
		"	122.9	3.2	13.5	90.1	13.6	144.9
8	Purina Dog Chow	Normal	141.6	8.8	19.2	109.8	23.1	165.6
	Non-protein	"	140.8	4.7	10.5	104.2	19.5	165.8
	Meat	"	161.2	25 3	23 5	124.0	22.1	164.0
9	Purina Dog Chow	"	157 8	8.5	16 7	118.3	22.7	164.5
	Non-protein	"	165.0	5.6	18.1	129 5	19.7	160.7
	Meat	"	151.0	24 4	26.2	126.7	19.7	166.7
10	Purina Dog Chow	"	170.0	10.1	19.1	132.4	20.3	165.0
	Non-protein	"	144.5	0.7	15.3	121.1	21.1	158.0
	Meat	"	156.0	18.2	20.1	123.9		

marked reduction in concentration of plasma bicarbonate, (2) practically no change in concentration of plasma fixed base, (3)

greatly increased excretion of fixed base in the urine so that the organism had actually lost a great deal of fixed base, and (4) greatly increased urine volume, amounting to a diuresis. The changes produced in Dogs 1 to 7 of Table I show the following comparison with the findings of Gamble. The percentage reduction in serum CO_2 capacity for Dogs 1 to 7 was respectively, 7.9, 14, 19, 24, 44, 42, and 29. In Dogs 2 and 3 there was no appreciable change in concentration of serum fixed base, in Dog 6 there was a 2.7 per cent reduction, and in Dogs 1, 4, 5, and 7 the percentage reduction ranged from 5.5 to 9.2. Furthermore, it was apparent to gross observation that Dogs 1 to 7 had a diuresis during the ingestion of fat or $CaCl_2$. In similar experiments when the urine was measured, the ingestion of $CaCl_2$ resulted in a 32 to 100 per cent increase in the 24 hour urine volume. The dogs also showed a marked reduction in plasma volume during the acidosis, inasmuch as a larger blood sample was necessary to secure sufficient serum for analysis. Because of these observations and the similarity of the changes in the serum CO_2 capacity and fixed base of Dogs 1 to 7 in Table I to those reported by Gamble there is no question but that these dogs have lost a great deal of fixed base. In fact they have lost so much that the mechanism which keeps the concentration of plasma fixed base rather constant in spite of other changes in blood electrolytes (7) has failed in five of the seven dogs. It seems perfectly logical to relate the reduction in concentration of fixed base in the succus entericus of Dogs 1 to 7 to this loss of base from the organism. Furthermore, it is of more significance that the reduction in fixed base of the juice occurred in dogs showing no change as well as small changes in concentration of serum base. This indicates either that the intestinal glands can lower the concentration of fixed base in the succus entericus in times of base deficit or that the rate of absorption of base from the intestinal contents has greatly increased, so that the juice as it escapes from the fistula is poorer in fixed base. Along with the reduction in fixed base there occurred a marked decrease in the concentration of chlorine. The tendency of the ratio of Cl to fixed base to increase in acidosis might be considered as additional evidence that the intestine possesses a mechanism for conserving base when there is a base deficiency. This reaction probably explains why the concentration of serum base undergoes less reduc-

tion than chlorine in intestinal obstruction (1, 8).   Furthermore, the report of Hoag and Marples (9) that the stool in diarrhea has less base in proportion to the acid ions than the normal stool suggests another illustration of this base-conserving mechanism of the intestine.

In later experiments it was learned that the protein intake in the diet had a marked effect upon the concentration of ammonia in the juice.   This is strikingly demonstrated in Dogs 8, 9, and 10. On a non-protein diet of starch and butter, the juice ammonia ranged from 0.7 to 5.6 milli-equivalents per liter and on Purina Dog Chow it ranged from 8.5 to 10.1 and on a high meat diet from 18.2 to 25.3 milli-equivalents per liter.   This variation apparently occurred independently of any variations in the fixed base of the succus entericus or blood serum.   The marked reduction in juice ammonia which occurred in Dogs 1 and 2 (Table I) was due, therefore, to a decrease in dietary protein.

Hydrogen Ion Concentration of Intestinal Contents—Although acidosis did not seem to excite the secretion of ammonia in the intestine, it was of some interest to see whether marked changes in the metabolism of the intestinal wall, as reflected in varying ammonia contents of the fistulous loop, would alter intestinal pH.

According to a recent review (10) of this problem, there is general agreement that the intestine is acid and that the acidity progressively decreases upon descending the intestine.   Graham and Emery (10) fed groups of dogs mixed diets, protein diet, fat diet, and carbohydrate diet for 2 weeks, at which time they were sacrificed.   The contents of various portions of the intestinal tract were removed and the pH determined colorimetrically.   The pH of the upper small intestine below the duodenum ranged from 6.0 to 6.7 and Graham and Emery concluded that their diets did not alter the intestinal pH.   Grayzel and Miller (11) fed groups of young dogs various types of diets such as normal, high carbohydrate, high fat, high protein, Mellanby's rachitogenic, and the rachitogenic plus either cod liver oil or ultraviolet irradiation. Samples of intestinal contents were taken 4 to 6 hours postprandially.   The intestine was acid throughout and no significant variation resulted with carbohydrate, fat, or protein diets.   The average pH range for the jejunum was from 5.8 to 6.59.   The rachitogenic diet caused a definite rise in the pH of intestinal .

contents to alkalinity. Ultraviolet irradiation or cod liver oil administration caused an acidification of intestinal contents to a normal range. Mann and Bollman (12), by means of surgical preparations which allowed direct access to various portions of the gastrointestinal tract in normal, conscious dogs, studied the pH of the intestinal contents, using the quinhydrone electrode. In the fasting animal, they found the contents of duodenum, jejunum, ileum, and colon to be alkaline, pH 7.0 to 8.0. The usual reaction in the small intestine after a meal was pH 6.5 to 7.5. The acidity of the intestine after a meal seemed to depend upon the development of acid in the stomach. De Beer, Johnston, and Wilson (4) found that the pH of juice from isolated jejunal loops in dogs ranged from 6.3 to 7.28 and in one dog it varied from 6.55 to 7.82. In a foot-note they state that calculation of CO_2 tension in the case of some of their data yielded values much higher than those for blood. Robinson (13) found that solutions after passing through Thiry-Vella fistulæ at various levels in the intestinal tract had a pH rather characteristic for that particular segment. For the jejunum the pH ranged from 6.2 to 6.7. Karr and Abbott (14) intubated the intestine of fasting human subjects. The intestinal contents were withdrawn under oil. They found the pH highly variable, ranging from 2.64 to 7.80. There was a tendency for the contents to become more alkaline lower in the intestines, although specimens of pH 4.8 were occasionally recovered even from the ileum.

In this study 87 pH determinations have been made of either the juice secreted by the jejunum or the jejunal contents in nine dogs. Representative data are shown in Table II. The contents included the isotonic NaCl-phosphate buffer which had passed through the Thiry-Vella fistula and succus entericus secreted in response to the mechanical stimulation. No more than one determination was made on any one dog in a day, so that the studies were spread over a considerable period of time. The succus entericus was always acid, the pH ranging from 5.62 to 6.80. The pH of the contents was approximately that of the juice alone. It ranged from 5.1 to 6.7 pH with 53 per cent of the values ranging between 6.0 and 6.7. When a hypertonic solution was passed through the fistula, the pH was higher, owing presumably to a movement of water and $BHCO_3$ into the intestine

TABLE II

Ammonia Content, pH, and CO₂ Tension in Jejunum of Dog.

Dog No.	Diet	pH	Total CO₂	Calculated CO₂ tension	Ammonia
			mM per l.	*mm. Hg*	*m.-eq.per l.*
10	Biscuit	6.52	9.14	84	4.5
	"	6.68	15.40	106	7.0
	"	6.40	9.65	107	7.1
	Purina Dog Chow	6.00	12.87	238	16.5
	" " "	5.83	6.29	136	15.2
	" " "	5.62	6.44	161	19.5
11*	Biscuit	6.1	15.73	261	3.5
	"	6.3	9.36	120	3.0
	Purina Dog Chow	5.6	9.82	247	9.5
	" " "	6.6	13 13	104	2.5
	" " "	6.52	14.02	129	17.0
	Meat	6.80	24 86	137	26.4
	"	6.58	21.14	154	30.5
12	Biscuit	6.00	10.86	201	2.2
	Purina Dog Chow	5.75	5.71	131	12.5
	" " "	6.20	14.49	213	8.0
13	Starch and butter	6.60	15 68	125	2.2
	" " "	5.70	11.77	279	1 6
	Purina Dog Chow	5.55	4.52	117	10 9
8	Starch and butter	6.55	13 27	115	3 5
	" " "	6.60	9.98	80	5.0
	Purina Dog Chow	6.00	6.38	118	
	" " "	6.70	4.58	30	7.0
	" " "	6.35	5.05	60	30.0
14	Biscuit	6.4	11.25	125	6 0
	"	6.7	7.41	49	4.5
	Purina Dog Chow	6.1	7.45	123	11.5
	" " "	5.7	5.21	123	24.7
15	Biscuit	6.57	12.12	101	6.1
	"	5.95	5.91	116	6.0
	Purina Dog Chow	5.70	3.72	88	12 0
	" " "	5.62	6.44	161	19.5
16	Starch and butter	6.45	8.94	132	5.4
	" " "	5.95	9.35	181	5.4
	Purina Dog Chow	5.85	5.34	113	10.3
	" " "	6.18	4 86	72	11.5

* All samples were succus entericus.

from the blood. For example, the pH of the jejunal contents with an isotonic buffer was 5.62 and with an 11 per cent NaCl solution the resultant pH was 6.15. When the intestine was more active as judged by the rate with which the buffer or succus entericus was propelled through the fistula, the pH of the succus entericus or jejunal contents was lower.

The CO_2 tension of juice and jejunal contents was calculated from the following arrangement of the Henderson-Hasselbalch equation

$$p = \frac{CO_2}{0.0591 \, \alpha \, (10^{pH-pK'} + 1)}$$

The value for α was taken as 0.51 and for pK' as 6.1. These assumptions seem justified inasmuch as the juice and the contents

TABLE III

Comparison of Succus Entericus from Dog, Collected under Oil and in Colostomy Bag

Dog No.	Method of collection	Total CO_2	pH	Calculated CO_2 tension	CO_2 lost on basis of increase in pH
		mM per l.		*mm. Hg*	*mM H_2CO_3*
11	Under oil	14.12	6.75	85	
	In bag	11.87	7.25	26	1.77
13	Under oil	21 35	6 50	202	
	In bag	15.12	7.20	36	5 0
16	Under oil	13.64	6.8	75	
	In bag	7 95	7.28	16	1.78

have about the same ionic concentration as that of blood serum (15). The succus entericus has about the same electrolyte content as blood serum, but only about 1 per cent protein. The calculated CO_2 tension ranged from 30 to 279 mm., with 70 per cent of the values between 60 and 140 mm. These data seem too high for normal physiology and yet in a physiological fluid containing $BHCO_3$ the acidity could only be due to the tension of CO_2.

A direct comparison was made with succus entericus collected under oil and in the colostomy bag. The second collection period directly followed the first. The data of Table III show that succus entericus collected in the bag loses more than enough CO_2 to account for the difference in pH. The lower CO_2 tension

seemed to correspond to a decidedly less active intestine.   A faster rate of secretion always resulted in juice of higher CO_2 tension. Campbell (16) has reported values of 36 to 63 mm. for CO_2 tension in the intestine.   McIver, Redfield, and Benedict (17) found by analysis of the residual gas after the introduction of oxygen or nitrogen into intestinal loops of anesthetized cats values for CO_2 corresponding to a tension of 36 to 47 mm.

The effects of diets containing various amounts of protein and no protein at all upon jejunal pH and CO_2 tension were compared (Table II).   The change from biscuit to Purina Dog Chow resulted in about a 50 per cent increase in urinary urea and about a 2- to 4-fold increase in the concentration of juice ammonia.   It was thought that at the higher concentrations of ammonia in the juice, the intestine might become less acid.   The diets of starch and butter or biscuit or Purina Dog Chow produced no consistent variations in pH and CO_2 values, although there were marked changes in ammonia content of the juice as seen especially in Dogs 8, 10, 14, and 15 when the ammonia content reached the level of 19.5 to 30 milli-equivalents.   Three dogs were fed all the lean meat they would eat.   In two dogs this amounted to a kilo daily.   The pH still remained within its usual range of acidity. Apparently, the protein diets do not cause the concentration of intestinal ammonia to become great enough to reduce the acidity. From these studies and those of other workers, it would seem that the intestinal pH is regulated chiefly by the metabolic activity of the digestive glands and epithelium lining the tract rather than by the comparative amounts of the various digestive products.

SUMMARY

1. Acidosis produced experimentally in dogs by a fat diet or administration of $CaCl_2$ and HCl resulted in a 6 to 32 per cent reduction of the concentration of fixed base in the succus entericus. With the exception of one dog, the concentration of fixed base in the juice in acidosis ranged from 15 to 42 milli-equivalents less than that of the corresponding blood serum.   This indicates that in times of base deficiency the intestine can conserve fixed base either by secreting a juice of lower base content or by increased absorption of base from the intestinal contents.

2. Acidosis did not increase the ammonia content of the juice,

although urinary ammonia had increased 2 to 9 times the normal. A reciprocal relationship between the concentrations of fixed base and ammonia in the succus entericus was never observed. Intestinal ammonia formation is not therefore an acid-base mechanism.

3. The ammonia content of the juice could be varied from 4.7 to 25.3 milli-equivalents per liter without materially altering the concentration of fixed base in either juice or serum by varying the amount of protein in the diet. In the earlier studies (1) of intestinal distention, the reduction in concentration of fixed base was an acid-base reaction on the part of the intestine, and the elevation in ammonia content was due to some other factors. These were two independent, fortuitous reactions.

4. The contents of succus entericus of the jejunum are acid, with most of the pH values ranging from 6.0 to 6.7. The corresponding CO_2 tension calculated from pH and total CO_2 ranged from 30 to 279 mm. of Hg, with 70 per cent of the values ranging from 60 to 140 mm. The high tension of CO_2 in the juice is believed to indicate that active tissues under physiological conditions may become very much more acid than any changes in the blood would indicate.

5. Diets which contained various amounts of protein, with the accompanying change in intestinal ammonia concentration, did not materially change the pH or CO_2 of the succus entericus or jejunal contents.

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THE DETERMINATION OF FUMARIC ACID IN PROTEIN SOLUTIONS CONTAINING SUCCINIC ACID

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In the light of the growing importance of the succinate-fumarate-enzyme system, there is a definite need for a suitable method of estimating fumaric acid in small amounts and in the presence of protein.

A method depending upon the partial oxidation of fumaric acid in amounts up to 1 mg. was recently described by Straub (1). The amount of permanganate required is not stoichiometrically equivalent to the fumaric acid oxidized; and the experimental ratio of the reactants found varies with the absolute amount of fumaric acid present. When this method is applied to protein solutions, there are large "blanks," even with extensive purification of the extractives.

In a second method by Hahn and Haarmann (2) fumaric acid is separated in amounts of approximately 500 mg. from succinic and malic acids by precipitation of the mercurous salt in 5 per cent HNO_3. The precipitate, washed with hot 5 per cent HNO_3, is weighed. The isolation of mercurous fumarate from a protein-containing solution involves several evaporations and extractions.

The principle of the latter method was adopted as the basis of the following micromethod. The present method is applicable to the determination of 2 to 12 mg. of fumaric acid with an accuracy of 3 to 5 per cent. The mercurous fumarate is estimated by oxidizing the mercurous to mercuric ions, and titrating the latter with thiocyanate. After examination of various factors which affected the accuracy of the method, the following procedure was adopted.

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Method

Precipitation of Proteins—A 4 ml. sample of the unknown solution containing protein is delivered into a 15 ml. conical centrifuge tube, treated with 0.5 ml. of 25 per cent trichloroacetic acid, and allowed to stand for 5 minutes. After the material is centrifuged and the supernatant fluid is transferred to a second tube, the precipitate is washed twice with 2 ml. portions of hot ether, and the washings are added to the original filtrate.

Precipitation of Barium Salts—A drop of saturated alcoholic methyl red is added, followed by alcohol until the whole is completely miscible. Hot saturated $Ba(OH)_2$ is added slowly until the red color changes to yellow. The tube is then completely filled with alcohol, thoroughly mixed, and allowed to stand for 30 minutes. After the contents are centrifuged and the supernatant fluid is removed, the precipitate is dissolved in 4 ml. of H_2O and 2 ml. of 15 per cent HNO_3.

Precipitation of Mercurous Fumarate—After the material is heated in a boiling water bath, 1 ml. of mercurous nitrate reagent is added, and the tube is simultaneously cooled and scratched until crystallization is induced. The tube is allowed to stand in the refrigerator for 2 to 4 hours (a longer time is inadvisable), then centrifuged for 5 minutes. After the supernatant fluid is drawn off, the precipitate is stirred with 2 ml. of 15 per cent HNO_3 and heated in a boiling water bath for a few minutes. After addition of 4 ml. of H_2O and 1 ml. of mercurous nitrate reagent, crystallization is again induced. (It may be noted that in all precipitations of mercurous fumarate the HNO_3 is present in a concentration of 5 per cent.) The tube is now allowed to stand in the refrigerator for at least 4 hours. After centrifugation, the precipitate is washed once with 5 ml. of 1 per cent $HgNO_3$ containing 5 per cent HNO_3, and twice with 5 ml. portions of 0.2 per cent HNO_3.

1 ml. of concentrated HNO_3 is added to the washed precipitate, stirred, and followed by approximately 5 ml. of H_2O. Saturated $KMnO_4$ is added in excess until the purple color persists, after which the tube is heated in a boiling water bath for a few minutes. After cooling, a minimum quantity of 3 per cent H_2O_2 is added to dissolve the MnO_2. The solution is quantitatively transferred to a 100 ml. Erlenmeyer flask, 2 ml. of ferric indi-

cator are added, and the mixture is titrated with standardized (approximately 0.02 M) ammonium thiocyanate.

Calculation—The ml. of NH_4SCN times its molarity, divided by 4, equals the mM of fumaric acid in the sample used.

Reagents—

Mercurous nitrate reagent. 10 per cent $HgNO_3$ in 5 per cent HNO_3.

Ferric indicator. Saturated ferric ammonium sulfate with HNO_3 added until the turbidity disappears.

Ammonium thiocyanate. Approximately 1.5 gm. are dissolved in 1 liter of H_2O and standardized with 0.02 M $AgNO_3$.

Hydrogen peroxide. A 3 per cent solution is prepared from 30 per cent superoxol.

The fumaric and succinic acids used in the development of the method were twice recrystallized. The melting point of the former was 285° (closed tube), of the latter 187°.

EXPERIMENTAL

Recoveries of 99 to 100 per cent were obtained by titrating 0.01 to 0.05 mM of mercuric nitrate in 5 per cent HNO_3 with standardized thiocyanate.

In order to arrive at the procedure described, the following factors were studied: (*a*) the solubility of mercurous fumarate and succinate in nitric acid, (*b*) the effect of the $HgNO_3$ concentration on the precipitation, and (*c*) the effect of recrystallization and higher acidity on the recovery. The recovery from solutions with and without protein was also studied.

Solubility of Mercurous Fumarate—It was found that successive washings of mercurous fumarate with 5 per cent HNO_3 caused a progressive decrease in the final yield of mercury. That this was due to solubility was also shown by the turbidity produced in the washings upon the addition of HCl. Mercurous fumarate was slightly soluble in 1 per cent HNO_3, but not in 0.2 per cent HNO_3. Since mercurous succinate was also insoluble in 0.2 per cent HNO_3, this was not a suitable washing reagent for removing succinate. The reagent chosen was 5 per cent HNO_3 containing 1 per cent $HgNO_3$, since it successfully repressed the solubility of mercurous fumarate, but dissolved mercurous succinate.

Effect of $HgNO_3$ Concentration—The result of precipitating

mercurous fumarate from solutions of fumaric acid and from a mixture of fumaric and succinic acids with different concentrations of $HgNO_3$ was studied. The tubes were allowed to stand in the refrigerator for 15 hours to insure complete crystallization, after which the precipitates were washed once with 5 ml. of 5 per cent HNO_3 + 1 per cent $HgNO_3$ and twice with 5 ml. portions of 0.2 per cent HNO_3.
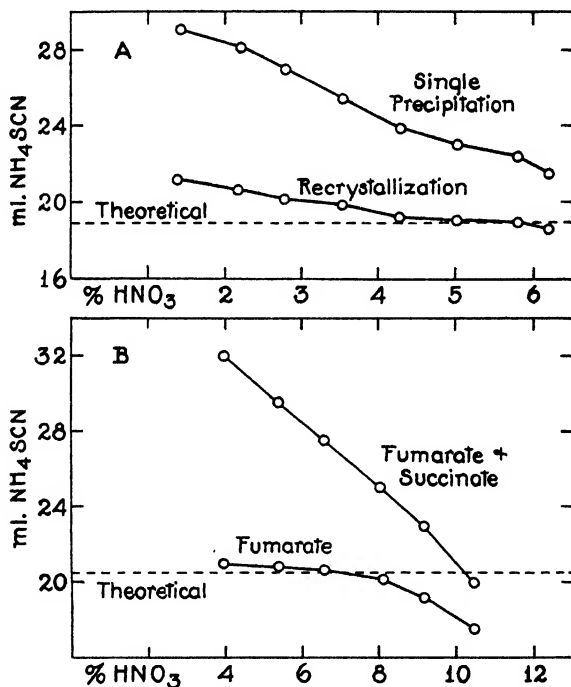
FIG. 1. A, effect of a recrystallization on the recovery of mercurous fumarate in the presence of succinate; B, effect of concentration of HNO_3 on the solubility of mercurous fumarate and succinate (without recrystallization).

It was found that 1 ml. of 10 per cent $HgNO_3$ was sufficient to precipitate completely 0.1 mM of fumaric acid, but that in the presence of succinic acid excessive amounts of mercury were recovered.

That this excess was due to mercurous succinate was shown in an experiment designed to study the time necessary for com-

plete precipitation.   The yield of mercury increased progressively
with the time allowed for crystallization only when succinate was
present.   Although mercurous succinate was qualitatively soluble
in 5 per cent HNO_3 + 1 per cent $HgNO_3$, the excessive mercurous
succinate could not be removed from the fumarate precipitate by
washing with this reagent alone.

The effect of recrystallization and varying HNO_3 concentration
on the recovery of fumarate in the presence of succinate was
studied.   It was found that precipitation and recrystallization

TABLE I

Recovery of Mercurous Fumarate from Solutions Containing Fumaric,
Succinic, and Malic Acids

Present in all experiments, 0.4 mM of succinic acid.

Fumaric acid	NH₄SCN		
	Theoretical	a	b
mM	*ml.*	*ml.*	*ml.*
0.100	17.24	17.38	17.36
		17.24	17.20*
0.075	12.93	13.09	13.00
		12.81	12.96
0.050	8.62	8.62	8.70
		8.74	8.70*
0.025	4.31	4.30	4.24
		4.41	4.28
0.015	2.58	2.56	2.46
		2.48	
0.010	1.78	1.06	1.22

* Indicates presence, in addition, of 0.4 mM of malic acid.

at acidities greater than 5 per cent HNO_3 eliminated contamina-
tion by mercurous succinate.   However, since at acidities greater
than 7 per cent, mercurous fumarate was found to be appreciably
soluble and difficult to crystallize, 5 per cent HNO_3 was chosen
as the solution providing the optimum conditions for separation
of the mercurous fumarate.   These facts are illustrated in Fig. 1.

Recovery from Pure Solution—Table I records the recoveries of
mercurous fumarate obtained from pure solutions of fumaric and
succinic acids, determined by the procedure described, but with
omission of the steps concerned with removal of proteins.

Recovery in Presence of Protein—Direct treatment of the filtrates following precipitation of the proteins by nitric or trichloroacetic acid did not yield mercurous fumarate under the conditions of precipitation described. Attempts to recover fumaric acid by evaporating acidified protein solutions or filtrates and extracting the residue with ether resulted in only 60 to 85 per cent recovery.

Successful recoveries were obtained, however, by making a trichloroacetic acid filtrate, extracting the precipitate with ether,

TABLE II

Recovery of Fumarate from Protein Solutions

Present in all experiments, 0.4 mM of succinic acid.

Fumaric acid	NH₄SCN	
	Theoretical	Found
mM	*ml.*	*ml.*
0.100	20 35	20.48
		20.62
		20.39
		20.50*
0.050	10.17	10.20
		10.26
		10.18
		10.42
0.015	3.05	3.00
		2.81
		2.86
		2.96*

* Indicates presence, in addition, of 0.4 mM of malic acid.

and adding alcohol until the whole was miscible. The barium salts of fumaric and succinic acids were then precipitated at pH 6.0 at which acidity the salts were insoluble. The barium salts readily dissolved in nitric acid, after which mercurous fumarate could be precipitated in the usual way.

The data given in Table II were obtained by analyzing solutions containing known amounts of fumaric acid, according to the procedure described under "Method." In addition to fumaric and succinic acids, the solutions initially contained tissue protein (succinic dehydrogenase preparation), phosphate, and methylene

blue. The two last named constituents were included in order to test the validity of the method under the conditions often encountered in the study of succinic dehydrogenase. The recovery of fumaric acid in such solutions was found to be satisfactory.

SUMMARY

A method for the determination of 2 to 12 mg. of fumaric acid in the presence of proteins and of succinic and malic acids has been described.

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THE COMPONENTS OF THE SUCCINATE-FUMARATE-ENZYME SYSTEM

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The relation of biological oxidations to reversible oxidation-reduction systems is commanding wide-spread interest. The present paper is an attempt to study the enzyme system which catalyzes the oxidation of succinic to fumaric acid from the standpoint of (1) certain properties of its essential components, and (2) of the effect produced on the system and its parts by reversible oxidation-reduction dyes.

The discovery of succinic dehydrogenase is attributed to Thunberg (1), when in 1909 he demonstrated that the oxygen consumption of washed minced muscle was increased by the addition of neutral succinate, and that succinate and washed muscle reduced methylene blue *in vacuo*. Widmark (2), Ohlsson (3), Andersson (4), Alwall (5), and Lehmann (6) have utilized skeletal muscle as a source of the enzyme, while recently Borsook and Schott (7) employed beef heart. Ogston and Green (8) prepared the enzyme from beef heart, utilizing a precipitation of the enzyme protein at its isoelectric point, and from liver by an ammonium sulfate precipitation.

Battelli and Stern (9), using oxygen as the H acceptor, first demonstrated that cyanide produced an inhibition of the oxidation of succinate by tissue. Thunberg (10), however, showed that cyanide did not inhibit the reduction of methylene blue. This could be interpreted that there were at least two essential moieties of the enzyme system, one of which had to do with oxygen activation and one with the activation of the hydrogen of the substrate. Szent-Györgyi, in 1924 (11), demonstrated this more clearly by showing that methylene blue could restore

the ability to consume oxygen to the cyanide-poisoned system.   He suggested that methylene blue reduction was an example of H activation, and the oxidation of p-phenylenediamine by succinic enzyme an example of O activation.

The functioning of both H activation and O activation in a succinic dehydrogenase preparation was not completely accepted, since as late as 1928 Wieland and Bertho (12) expressed the belief that cyanide was simply adsorbed on the surface of the dehydrogenase, and that methylene blue was able to displace it.

The O-activating properties of the preparation seem to be dependent on the presence of Keilin's indophenol oxidase and of cytochrome.   With the exception of the preparations of Ogston and Green (8) succinic enzyme systems have contained both of these factors (Keilin (13))—in fact, the simple phosphate extraction of washed tissue is one of the best known methods at present of obtaining indophenol oxidase.

Preparation of Enzyme

The method of preparing the enzyme used in this study was, with minor modifications, essentially that described by Borsook and Schott (7).

100 gm. of fat- and fascia-free beef heart were twice ground in a meat grinder.   The tissue was placed on a large piece of muslin and put in a 2 liter evaporating dish half filled with water at 40–45°.   It was washed and pressed occasionally by hand for 10 minutes, and the wash water discarded after the juice was pressed from the muscle mass.   The operation was repeated with cold water for the same length of time.   This was continued, making a total of four hot and four cold water washings.   It was then ground thoroughly with an equal volume of sand and 200 ml. of $M/15$ K_2HPO_4.   After the mixture had become a light brown homogeneous paste, it was allowed to stand for 30 minutes with occasional stirring.   The mixture was centrifuged for 20 minutes and the supernatant fluid filtered through muslin.

The activity of the preparation was tested by the Thunberg technique, which has been standardized in the case of succinic dehydrogenase by Lehmann (6).   The composition of the reaction mixture used in this test was 2.0 ml. of $M/15$ Sørenson phosphate buffer (pH 7.3), 0.2 ml. of 0.2 M neutral succinate, 0.3 ml.

of methylene blue (4×10^{-4} mM), and enzyme solution, 0.5 cc.
The decoloration times at 37° *in vacuo* for enzymes prepared as
described above ranged from 6 to 11 minutes. Actually this
test is a measure only of the "dehydrogenase factor" (H-activat-
ing) of the enzyme and does not necessarily parallel its activity
in the presence of oxygen.

Methods of Measurement

The oxygen consumption was measured manometrically in
Erlenmeyer type Warburg vessels with side arms at 37° $\pm$ 0.05°.
The reaction vessels contained a total of 3 ml. This usually
consisted of 0.5 ml. of enzyme preparation, 1.5 ml. of M/15 phos-
phate buffer (pH 7.35), 0.3 ml. of 0.2 M succinate in the side arm,
and water to 3 ml. Deviations from these amounts will be noted
in the appropriate tables. Readings of the manometers were
made every 10 minutes for at least 60 minutes. Unless otherwise
specified, oxygen consumptions will be reported as c.mm. of oxy-
gen consumed per hour. The determination of fumaric acid was
carried out by the method described in the preceding paper (14).

Experiments were run in duplicate and repeated with at least
two different enzyme preparations for each point studied.

Comparison of Oxygen Consumption and Fumarate Production

Fischer (15) concluded that in the action of washed muscle on
succinate there was produced an equilibrium mixture of one-
third fumaric acid and two-thirds malic acid. Alwall (5) showed,
however, that, if the ground muscle was washed with warm water
before phosphate extraction, fumarase was eliminated.

This procedure was employed in our method of preparation,
but in order to determine whether fumarase had been removed,
a series of experiments was carried out in which the oxygen con-
sumed in the oxidation of succinate was compared with the
amount of fumarate formed. If these two determinations
agreed, evidence would be provided that only fumarate resulted
from the oxidation of succinate and consequently that fumarase
was absent.

The comparison of oxygen consumption and fumarate produc-
tion was studied in two ways: (Method A) by shaking an identical
mixture of the reactants in separate vessels at the same rate and

temperature as those in the Warburg vessels and removing duplicate samples for fumarate analysis simultaneously with the reading of the manometer; (Method B) by allowing the reaction to proceed to completion in the Warburg vessel, after which the

TABLE I

Comparison of Fumaric Acid Production and Oxygen Consumption

pH 7.35; temperature 37°; total succinate 0.072 mM.

Method	Fumarate found	O₂ equivalent	O₂ consumed
	mM	*c.mm.*	*c.mm.*
A	0.0599	671	683
"	0.0650	727	720
"	0.0689	772	795
B	0.0698	782	812*
"	0.0688	770	808*

' Theoretical for 0.072 mM of succinate   ᵢ c.mm.

TABLE II

Rate of Oxidation of Succinate by Succinic Enzyme

pH 7.36; temperature 37°; total succinate 0.072 mM.

Time	Oxidation	$k = \frac{1}{t} \log \frac{a^*}{a-x}$	Time	Oxidation	$k = \frac{1}{t} \log \frac{a}{a-x}$
min.	*per cent*	$\times 10^{-4}$	*min.*	*per cent*	$\times 10^{-4}$
5	7.9	70.7	60	71.0	89.6
10	17.5	82.8	70	76.3	89.2
15	25.4	84.6	80	80.2	88.0
20	32.4	85.2	90	83.6	87.3
25	40.2	89.1	100	85.3	83.3
30	45.6	88.4	120	89.6	82.0
35	50.8	88.0	140	92.8	81.5
40	55.7	88.5	160	95.2	82.5
45	60.5	89.5	190	97.8	87.3
50	64.7	90.6	215	98.9	91.0
55	67.8	89.4	Average...........		88.0

* a denotes initial amount of succinate (0.072 mM) taken as 100; x denotes per cent of succinate oxidized; t denotes time in minutes.

sample was removed for analysis of the fumarate formed. The mM of fumarate were expressed in terms of the c.mm. of oxygen used to form it, and compared with the actual oxygen consumption. The results are recorded in Table I.

It was concluded from these data that our succinic dehydrogenase preparations did not contain appreciable amounts of fumarase, since fumaric acid was recovered in amounts equivalent to the oxygen consumed.

Rate of Oxidation of Succinate

The velocity constants of the oxidation of succinate were calculated in three experiments which were carried to completion. The results of one such experiment are given in Table II. It will be seen that under the conditions of our experiment, and in the presence of an excess of oxygen, the rate of oxidation may be expressed as a monomolecular reaction. There was little deviation from the average constant of 88×10^{-4} except for the

TABLE III

Relation between Varying Amounts of Enzyme and Oxidation of Succinate in Presence of NaCN and Methylene Blue

pH 7.35; temperature 37°; NaCN 0.006 mM; methylene blue 10×10^{-4} mM.

Time	O_2 consumption		
	Enzyme 0.2 ml.	Enzyme 0.4 ml.	Enzyme 0.6 ml.
min.	*c.mm.*	*c.mm.*	*c.mm.*
20	82	129	181
40	151	221	294
60	194	275	347

last 15 per cent of the oxidation, during which some inhibition of the reaction occurred.

Method of Studying Dehydrogenase Factor

The dehydrogenase factor in the enzyme preparation may be defined as the activator of the hydrogen of the succinate, which may then be oxidized by a hydrogen acceptor; *e.g.*, methylene blue or activated oxygen. This factor may be measured by the methylene blue reduction time, but the difficulty of observing accurately the time of complete decoloration makes this method inaccurate. If the oxidizing catalyst (O-activating) were poisoned (*e.g.* NaCN) and replaced by a constant amount of an artificial catalyst (*e.g.* methylene blue) in excess, then the H-

activating agency should be the limiting factor in the reaction as measured by oxygen consumption methods.

In testing this experimentally with 0.06 mM of succinate, 0.006 mM of NaCN, and 0.001 mM of methylene blue, it was found that the oxygen consumption was decreased with decreasing amounts of enzyme. Sample results of such an experiment are given in Table III. The velocity constants calculated for this experiment were found to be 31×10^{-4}, 45×10^{-4}, and 65×10^{-4}, corresponding to enzyme quantities of 0.2, 0.4, and 0.6 ml. respectively. The degree to which the activity of the enzyme is changed in the presence of a constant amount of NaCN and artificial catalyst will be used in this paper as a measure of the degree to which various agencies affect the H-activating portion of the enzyme preparation.

TABLE IV

Relation between Varying Amounts of Enzyme and Oxidation of Phenylenediamine

pH 7.35; temperature 37°; phenylenediamine 0.5 ml. of 1 per cent.

Time	O$_2$ consumption		
	Enzyme 0.2 ml.	Enzyme 0.4 ml.	Enzyme 0.6 ml.
min.	*c.mm.*	*c.mm.*	*c.mm.*
20	71	211	383
40	140	367	531
60	202	471	617

Method of Studying Oxidase Factor

Indophenol oxidase (Keilin) has long been considered the agency in heart muscle extracts which oxidizes p-phenylenediamine. This substance does not itself require "activation" but is oxidized by active oxygen. Upon oxidation, the colored quinonediimide is formed, with the consumption of oxygen. Fieser (16) has shown that this system is not strictly reversible, since the first oxidation product combines further with unused phenylenediamine to produce complexes.

An example of the results found upon increasing the amount of enzyme on the rate of oxidation of phenylenediamine is recorded in Table IV. The velocity constants of this reaction could not be calculated owing to the uncertainty regarding the

final oxidation product. The data for this reaction are therefore recorded in terms of oxygen consumed. Since the rates are dependent upon the amount of enzyme present, this test was used to study the effect of substances on the oxidase portion of the enzyme.

If the oxygen activation were entirely through the agency of "indophenol oxidase," then the phenylenediamine oxidation and

TABLE V

Percentage Inhibition of Phenylenediamine and Succinate Oxidation
pH 7.35; temperature 37°; time 30 minutes.

NaCN	Inhibition	
	Succinate oxidation	Phenylenediamine oxidation
*m*M	*per cent*	*per cent*
0.0002	71	64
0.0003	80	86
0.0005	88	90
0.0007	91	98

TABLE VI

Effect of Poisons and Aging on Components of Enzyme System
pH 7.35; temperature 37°.

Inhibiting agent		Inhibition	
		Oxidase	Dehydrogenase
	*m*M	*per cent*	*per cent*
Cyanide....	0.006	100	0
Selenite	0.02	0	100
Urethane.. :................... ..	1.8	26	30
Fluoride...	0.06	16	45
Pyrophosphate	0.06	38	0
7 days standing at 10°........ . ..		53	13

the succinate oxidation should be equally sensitive to cyanide. With this in mind, the percentages of inhibition caused by increasing amounts of cyanide were compared. The results of such a comparison are given in Table V. Since the inhibition is approximately the same in the two instances, these experiments suggest that a cyanide-sensitive oxidase is responsible for oxygen activation in this system.

Effect of Poisons and Aging on Enzyme Factors

By the methods outlined, a few typical enzyme poisons and the effect of aging of the enzyme were examined. Examples of resulting inhibitions of the two components of the system are summarized in Table VI.

Cyanide and selenite are notable in that they cause a complete inhibition of one of the factors without affecting the other.

The statements so commonly seen in the earlier literature concerning the stability of the enzyme actually apply to the dehydrogenase component, since the activity was measured by

TABLE VII

Effect of Heat on Components of Enzyme System

pH 7.35; temperature 37°; time of heating 45 minutes.

Reactants	O₂ consumption	
	30 min.	50 min.
	c.mm.	*c.mm.*
Enzyme + phenylenediamine............................	210	285
" heated at 55° + phenylenediamine................	131	190
" " " 65° + "	5	8
" " " 75° + "	0	0
" " " 55° + succinate........................	0	0
" " " 55° + " + cresyl blue.........	0	0
" + succinate + NaCN + cresyl blue.............	185	225
" heated at 55° + succinate + NaCN + cresyl blue	0	0
" " " 55° + " + " * + " "		
+ additional untreated enzyme..................	175	228

* The NaCN used here was sufficient to poison all the oxidase present.

methylene blue reduction. Actually the oxidase activity is considerably decreased by storage, but the dehydrogenase component is quite stable.

Barron and Hastings (17), in studying the components of their lactate-oxidizing enzyme, also concluded that storage produced greater destruction of the oxidase component.

Stability of Enzyme to Heat

Portions of the enzyme were heated in a water bath at various temperatures for 45 minutes, and the properties of the resulting

solutions compared with those of the unheated enzyme. The results are summarized in Table VII.

Since the heated enzyme preparation was unable to oxidize succinate in the presence of cyanide and cresyl blue (artificial oxidizing catalyst), it was concluded that the dehydrogenase factor was completely destroyed under the conditions of this experiment. The effect of heat on the phenylenediamine oxidation shows that the oxidizing enzyme was only partially inactivated by heating at 55° for 45 minutes, almost completely destroyed at 65°, and entirely inactivated at 75°. Final proof of the dehydrogenase destruction was obtained by adding dehydrogenase in the form of cyanide-treated enzyme, which again established succinate oxidation.

Barron and Hastings (17) utilized 55° heating for a separation of the two factors in their lactate-oxidizing enzyme, and concluded that the "oxidase" component was destroyed. Although there is no ·proof that their "oxidase" is identical with that operating in the succinate system, there would appear to be some difference between the lactic and succinic enzyme systems. Ahlgren (18) found that succinic dehydrogenase was inactivated at 55° as tested by methylene blue reduction. Keilin (19) states that indophenol oxidase is destroyed at 70° and that dehydrogenase is destroyed at 52°. He makes the statement, however, that after heating (yeast) to 52° "only lactate had a very marked effect in accelerating the reduction of oxidized cytochrome. The effect of sodium succinate and pyruvate is less marked." This would suggest that lactic dehydrogenase is more stable to heat than succinic dehydrogenase and offers an explanation of the apparent discrepancy noted above.

Effect of Reversible Oxidation-Reduction Dyes

We have compared the effect of several dyes of different oxidation-reduction potentials on the succinate and phenylenediamine oxidation and their catalytic effect on the system when it is either partially or completely deprived of oxidizing enzyme. The dyes used in these experiments are given in Table VIII.

Effect on Succinate Oxidation—Meyerhof (20) found that methylene blue usually produced an inhibition of succinate oxidation by washed muscle and muscle extracts. Wieland and

Frage (21) also demonstrated a slight inhibition by methylene blue on succinate oxidation by dried muscle powder. Ogston and Green (8), however, found that this dye produced an acceleration of succinate oxidation. Their enzyme was prepared by ammonium sulfate precipitation and the succinate oxidation was greatly increased by cytochrome. This indicates that their preparation was more deficient in oxidizing catalyst than the usual succinic dehydrogenase.

TABLE VIII

Reversible Oxidation-Reduction Dyes Used

Dye	E'_0 at pH 7.3
	volt
2,6-Dichlorophenol indophenol....................	+0.200
o-Cresolindo-2,6-dichlorophenol...	+0.155
Naphtholsulfonate indophenol............................	+0.105
Thionine.....	+0.045
Cresyl blue..	+0.020
Methylene blue	−0.005
Indigotetrasulfonate	−0.065
Succinate-fumarate.	−0.020

TABLE IX

Éffect of Methylene Blue on Succinate Oxidation

pH 7.36; temperature 37°; succinate 0.06 mM.

Methylene blue	O_2 consumption at 60 min.	$k = \frac{1}{t} \log \frac{a}{a-x}$
$m_M \times 10^{-4}$	*c. mm.*	$\times 10^{-4}$
0	442	82
1.87	433	71.5
3.75	393	62.0
7.5	308	42.5

In our experiments greater inhibition of oxidation was produced by increasing amounts of methylene blue. Table IX records the oxygen consumptions at 60 minutes obtained with various amounts of methylene blue. The velocity constants of the reaction were calculated and found to bear a linear but inverse relation to the concentration of methylene blue.

Equimolar amounts of different dyes were also tested for their

effect on the oxidation. The results of one such experiment are recorded graphically in Fig. 1.
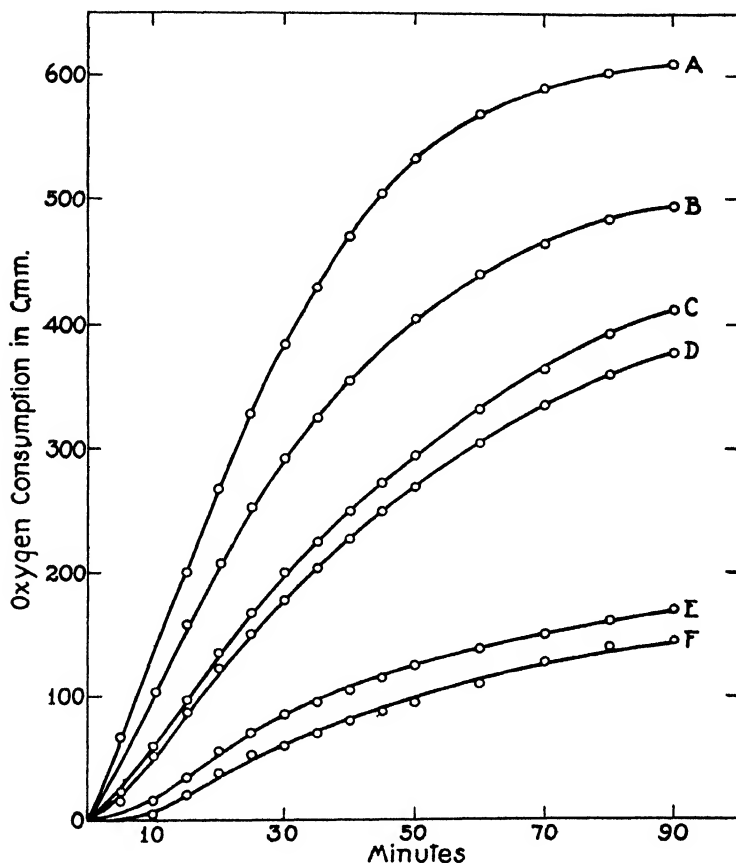
FIG. 1. Effect of dyes on succinate oxidation. Dye, 6×10^{-4} mM; pH, 7.35; T, 37°. Curve A, without dye; Curve B, methylene blue; Curve C, 2,6-dichloronaphtholsulfonate indophenol; Curve D, naphtholsulfonate indophenol; Curve E, o-cresolindo-2,6-dichlorophenol; Curve F, 2,6-dichlorophenol indophenol.

Obviously these dyes all cause an inhibition of the succinate oxidation, and apparently the more positive the potential of the dye the greater the inhibition. Thus, methylene blue whose

potential (E'_0 at pH 7.3, -0.005 volt) is close to that of the succinate-fumarate system (E'_0 at pH 7.3, -0.02 volt) produced an inhibition of 24 per cent at 30 minutes, while 2,6-dichlorophenol indophenol caused an inhibition of 84 per cent. However, an investigation of the effect of dyes whose potentials were between 0.00 and $+0.05$ volt showed that cresyl blue (E'_0 at pH 7.3, $+0.020$ volt) with a potential higher than methylene blue caused little or no inhibition of the succinate oxidation; and thionine (E'_0 at pH 7.3, $+0.045$ volt) with a still higher potential caused an inhibition equal to that of methylene blue. Table X records the results of one such experiment. The significance of these results will be discussed later.

No definite explanation is offered for the inhibition caused by

TABLE X

Effect of Dyes on Succinate Oxidation

pH 7.33; temperature 37°; dye 6×10^{-4} mm.

Dye	O_2 consumption at 60 min.
	c. mm.
None...	560
Methylene blue...........................	507
Cresyl blue......	558
Thionine.....................................	488

the dyes, but that they enter into the reaction is suggested by the following observations: (*a*) 2,6-dichlorophenol indophenol became partially decolorized during the course of the reaction, but the lower potential dyes remained visibly in the oxidized form; (*b*) all of these dyes were reduced *in vacuo* by the enzyme system, and the decoloration time was not affected by cyanide; (*c*) all of the leuco dyes could be reoxidized by a factor in the enzyme preparation which was cyanide- and heat-sensitive.

The latter conclusion was reached by observation of the time necessary for half oxidation of the leuco dye after air was admitted to the decolorized mixture. In each case, the dye was oxidized much faster in the absence of cyanide than in its presence. Heating the decolorized mixture at 90° for 20 minutes before admitting air also increased the recoloration time.

These facts indicate strongly that the dyes enter into the reaction and that their rôle is related to their oxidation-reduction potentials.

Effect of Dyes on Phenylenediamine Oxidation—In order to evaluate the effect of the dyes on the oxidase factor, the oxidation of phenylenediamine was studied. The experiments showed that the high potential dyes had no marked effect on the oxidation, but that the dyes of lower potential at a high concentration had a slight inhibitory effect. Table XI records typical results of this kind.

The relation between potential of the dye and inhibition observed in these experiments is qualitatively different from the

TABLE XI

Effect of Dyes on Phenylenediamine Oxidation

pH 7.35; temperature 37°; phenylenediamine 0.5 cc. of 1 per cent.

Dye	O_2 consumption at 60 min.	
	6×10^{-4} mм dye	1×10^{-3} mм dye
	c.mm.	*c.mm.*
None	478	602
Methylene blue	425	338
Cresyl blue	466	468
Thionine	458	554
o-Cresolindo-2,6-dichlorophenol	472	582
2,6-Dichlorophenol indophenol	481	562

effect of the same dyes on succinate oxidation. It is therefore improbable that the action of the dyes on the oxidase factor accounts for their inhibiting action on the succinate oxidation. It is recognized, however, that this experiment is not conclusive, since the effect of the dyes may be on phenylenediamine itself, or on its oxidation products.

Effect of Dyes in Replacing Oxidase

Of the dyes studied, only those with potentials positive to that of the succinate-fumarate system were able to cause succinate oxidation by the cyanide-poisoned enzyme system. In this case the degree of restoration was proportional to the amount of dye

added. In Fig. 2 this is illustrated in the case of methylene blue with a partially poisoned enzyme system.
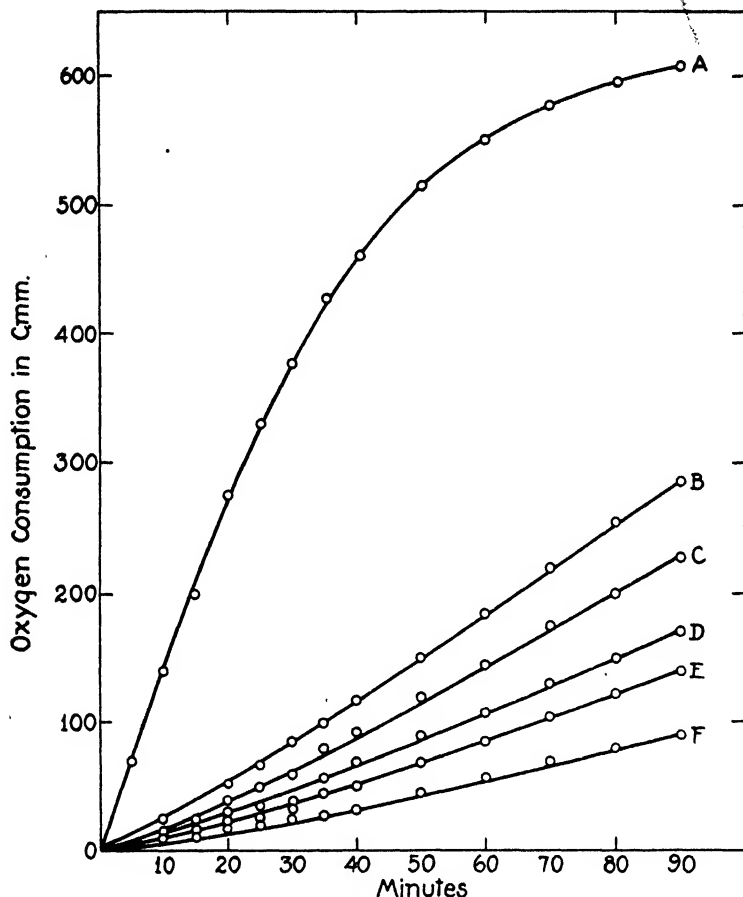
FIG. 2. Effect of methylene blue on the partially poisoned enzyme system. pH, 7.36; T, 37°; succinate, 0.06 mM. Curve A, methylene blue 6×10^{-4} mM; Curve B, methylene blue 6×10^{-4} mM $+ 0.001$ mM NaCN; Curve C, methylene blue 3×10^{-4} mM $+ 0.001$ mM NaCN; Curve D, methylene blue 2×10^{-4} mM $+ 0.001$ mM NaCN; Curve E, methylene blue 1×10^{-4} mM $+ 0.001$ mM NaCN; Curve F, none $+ 0.001$ mM NaCN.

The degree of restoration produced by equimolar amounts of different dyes again showed a relation to their potentials. Table

XII records the oxygen consumption found upon the addition of different dyes to the completely poisoned system.

It will be noted here again that cresyl blue gave rise to the greatest restoration of oxygen uptake, just as it inhibited the unpoisoned succinate oxidation the least. Likewise, thionine, in spite of being considerably higher in potential than methylene blue, restored succinate oxidation to a greater extent. This was true in other experiments involving different amounts of the dyes.

It is thought that in these experiments there was simultaneous

TABLE XII

Effect of Dyes in Restoring Succinate Oxidation after Cyanide Poisoning

pH 7.36; temperature 37°; 0.006 mM NaCN present in all except where noted by the asterisk.

Dye	mM × 10^{-4}	E'_0 at pH 7.3	O_2 consumption at 60 min.
		volt	*c.mm.*
None*................................			567
"			2
Methylene blue.........................	6.6	−0.005	294
Naphtholsulfonate indophenol...........	6.6	+0.105	39
o-Cresolindo-2,6-dichlorophenol.........	6.6	+0.155	25
2,6-Dichlorophenol indophenol...........	6.6	+0.200	13
None*................................			377
Methylene blue	3	−0.005	105
Cresyl blue............................	3	+0.020	257
Thionine..............................	3	+0.045	157
Naphtholsulfonate indophenol...........	3	+0.105	22

reduction of the dye by the enzyme system and oxidation of the leuco dye by atmospheric oxygen. At the end of the experiment it was found that 2,6-dichlorophenol indophenol was completely reduced, o-cresolindo-2,6-dichlorophenol only partially oxidized, and the others largely oxidized. This would indicate that in the case of the high potential dyes the rate of reduction exceeded the rate of autoxidation of the dye. Dyes of potential lower than the succinate-fumarate system (*e.g.* the indigosulfonates) and which were completely oxidized throughout the experiment, were unable to catalyze the cyanide-poisoned system.

Effect of Mixtures of Dyes on Oxidation

In view of the possibility of several reversibly oxidizable substances of different potentials coexisting in the cell, it was of interest to note the effect of two dyes of different potential on the succinate oxidation, both cyanide-poisoned and unpoisoned. An example of such an experiment is given in Table XIII. It was found that a dye of higher potential decreased the oxygen uptake established by one of lower potential, and further suggests that the degree of inhibition is related to the potential of the dye added.

Since it has been shown that the oxidation of leuco methylene

TABLE XIII

Effect of Mixture of Dyes on Succinate Oxidation

pH 7.35; temperature 37°; 0.06 mM succinate; total amount of each dye 3×10^{-4} mM.

Dye	E'_0 at pH 7.3	O$_2$ consumption at 60 min.	
		No cyanide	0.006 mM NaCN
	volt	*c.mm.*	*c.mm.*
Methylene blue	-0.005	565	224
" " and naphtholsulfonate indophenol	-0.005 $+0.105$	412	120
Methylene blue and o-cresolindo-2,6-dichlorophenol..................................	-0.005 $+0.155$	92	87

blue by air is a typical autoxidation with the formation of a peroxide (22), the effect of the second dye noted here may be an example of antioxidation.

Incomplete Enzyme

An enzyme was prepared in the usual way except that the preliminary washing of the tissue was doubled in amount. This was done in order to determine whether there would appear any qualitative differences in the effect of dyes upon the reaction.

This preparation differed from the usual one in possessing lower activity for the oxidation of succinate by oxygen in spite

of a high oxidase content and dehydrogenase activity. Furthermore, the oxidation of succinate was accelerated instead of inhibited by dyes whose E'_0 varied between 0.00 and $+0.05$ volt. These results suggest that a substance essential for the interaction of the dehydrogenase and the oxidase was removed by additional washing. In the light of Ogston and Green's work (8), such an intermediate might have been cytochrome.

The properties of this enzyme offered another opportunity to study the relation of the dye potentials and their effect on the succinate oxidation. The results obtained with a series of dyes are recorded in Table XIV and show that the dyes exerted their effect in the same order as was found on the whole enzyme and cyanide-poisoned enzyme systems.

TABLE XIV

Effect of Dyes on Succinate Oxidation by Incomplete Enzyme
pH 7.35; temperature 37°.

Dye	E'_0 at pH 7.3	O₂ consumption at 60 min.
	volt	*c.mm.*
None...		206
Methylene blue.............................	-0.005	228
Cresyl blue..................................	$+0.020$	288
Thionine.....................................	$+0.045$	224
Naphtholsulfonate indophenol.................	$+0.105$	147
o-Cresolindo-2,6-dichlorophenol..............	$+0.155$	107

DISCUSSION

The experiments reported here indicate that for the enzymic oxidation of succinate by oxygen, there must be present two essential parts: (a) the dehydrogenase factor, which is completely inhibited by selenite and is not replaceable by dyes, and (b) the oxidase factor, which is completely inhibited by cyanide and is replaceable by reversible dyes, the extent of the replacement depending upon their potentials. The latter factor is capable of activating oxygen which may then accept hydrogen of the substrate which has been activated by the dehydrogenase.

The failure to observe a catalytic action of dyes in a fresh enzyme system has been previously noted by Barron and Hastings

(17) in the case of the lactate-oxidizing system.   The present experiments indicate that the dyes may play an integral part in succinate oxidation in being reduced by the dehydrogenase and substrate, and oxidized by the oxidase factor.   In the preparation in which a part of the oxidase factor was apparently lacking, the dyes whose E'_0 are between 0.00 and +0.05 volt showed an acceleration of the oxidation.   In a third case, in which the oxidase
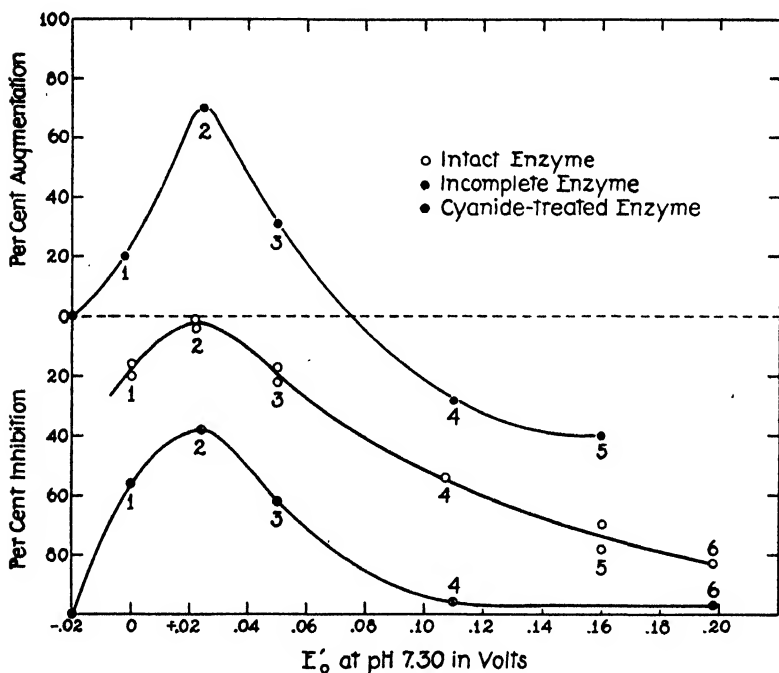
FIG. 3. Effect of dyes on activity of succinic enzyme. *1*, methylene blue; *2*, cresyl blue; *3*, thionine; *4*, naphtholsulfonate indophenol; *5*, o-cresolindo-2,6-dichlorophenol; *6*, 2,6-dichlorophenol indophenol.

factor was completely poisoned and oxidation of the dye depended entirely upon autoxidation, similar relations of the dyes were found.   These results are summarized graphically in Fig. 3.   It should be noted that the extent to which the lowest curve rises above the base line denotes the degree to which the oxidative activity of the enzyme had been restored.

It is evident that in three different cases there was a correlation of the effect of the dyes and their potentials. The least inhibition, or the greatest augmentation occurred in each case with cresyl blue. Furthermore, thionine in each case had an effect comparable with methylene blue in spite of being considerably higher in potential.

Such a finding may be expected to exist in view of the work of Barron (23) and of Voegtlin, Johnson, and Dyer (24). The former found a linear relation between the E'_0 of a series of dyes and the logarithm of the time necessary for half oxidation of the dye, dyes of lower potential autoxidizing the faster. The latter workers, in studying the reduction of oxidation-reduction indicators by tissues, concluded that the "reduction time is approximately a logarithmic function of the electrode potential." It is believed that in the experiments described in this paper the dyes used have exerted their effects by virtue of the relative velocities with which they were reduced and oxidized, which in turn is related to the potentials of the dyes.

It is of interest that we have found, in a simple system, a phenomenon similar to that observed by Barron and Hoffmann (25) in their study of the catalytic effect of reversible dyes on the metabolism of starfish eggs. They found that dyes whose normal potentials were between 0.00 and +0.10 volt were most effective in accelerating the metabolism of the eggs. Dyes whose potentials were higher or lower than this were less effective accelerators. Their results were complicated, however, by differences in the diffusion rate and toxicity of the dyes. In addition to this work, Barron and Hastings (17) demonstrated the high catalytic power of nicotine-hemin as compared to hemin on the lactic enzyme system and suggest that this effect was due to the more favorable potential of the hemochromogen. Finally, the results of Elliott and Baker (26), on the effect of dyes on tumor metabolism in the presence of glucose, suggest a grading effect, from inhibition with high potential dyes to acceleration with dyes of potential similar to that of methylene blue.

It seems reasonable to conclude, therefore, from the experiments presented in this paper, that reversibly oxidizable systems exert a quantitative influence on the rate of oxidation of activated substrates; and furthermore that the nature and extent of their

influence are determined by the oxidation-reduction potential of the reversible system.

SUMMARY

1. A succinic dehydrogenase was prepared which contained no fumarase, as tested by a comparison of oxygen consumption and direct fumarate determinations.

2. The rate of oxidation of succinate by this enzyme followed a monomolecular course.

3. The enzyme was shown to consist of two factors. One of these is responsible for the specific activation of the succinate molecule; the other activates molecular oxygen. Tests were described for the respective factors, and the effect of various agencies upon them examined.

4. The relation of the potentials of dyes and their effect on the whole enzyme system and its parts was studied and discussed.

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CONTRIBUTIONS TO THE STUDY OF MARINE PRODUCTS

V. THE PRESENCE OF STIGMASTEROL IN MOLLUSKS

By WERNER BERGMANN

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In a previous communication it has been demonstrated (1) that the typical sterol of the oyster, *Ostrea virginica*, is ostrea-sterol. When the crude bromoacetate of this sterol is treated with ether, all but a small crystalline residue goes into solution. On one occasion several hundred mg. of this insoluble substance were obtained, which made its identification possible. The re-crystallized material melted at 199–200° and showed $[\alpha]_D^{20} = -40.71°$. It contained bromine and proved to be a tetrabromide of the formula $C_{31}H_{50}O_2Br_4$. These data corresponded very closely to those of tetrabromostigmasteryl acetate (2), and a mixture of both substances failed to show a depression of the melting point. The acetate, obtained by debromination, was also found to be identical with stigmasteryl acetate. Stigmasterol, therefore, is a constituent of the oyster.

While this work was in progress, Fernholz in a private communication informed the author that the sterol mixture obtained from the common mussel, *Mytilus edulis*, contains about 5 per cent stigmasterol, which had been identified as the tetrabromo-acetate.

The stigmasterol content of the oyster is not constant, but apparently undergoes seasonal variations. Sometimes it is present in such small quantities that it can hardly be detected, while occasionally it has been found to represent as much as 4 per cent of the total sterol.

Heilbron (3) has recently shown that fucostanol, ostreastanol (4), sitostanol, and stigmastanol are identical. Ostreasterol, therefore, differs from stigmasterol only in the position of the

double bonds. In contrast to the latter, which possesses a double bond at C_{22} in the side chain (5), the double bonds of ostreasterol are contained in the ring system, for I have found during an as yet unpublished investigation that ostreasterol fails to give ethyl-isopropylacetaldehyde (6) or a similar product on treatment with ozone.

<div align="center">EXPERIMENTAL</div>

Isolation of Tetrabromostigmasteryl Acetate—The crude bromo-acetate of ostreasterol was treated with 10 times its weight of ether. The residue was filtered, washed with a few drops of ether, and recrystallized several times from chloroform and alcohol; m.p., 199–200°.

Rotation—0.0451 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of $-0.612°$, hence $[\alpha]_D^{20} = -40.71°$.

Analysis—3.209 and 3.077 mg. of substance gave 1.325 and 1.269 mg. of Br. Calculated for $C_{31}H_{50}O_2Br_4$, Br 41.32, found Br 41.30 and 41.25.

Mixed with tetrabromostigmasteryl acetate, m.p. 200–201° and $[\alpha]_D^{21} = -40.2°$, it did not show a depression of the melting point.

Stigmasteryl Acetate—The bromoacetate was debrominated by refluxing it with zinc dust in glacial acetic acid for 4 hours. After that time the solution was filtered and distilled water was added to the warm solution until it turned slightly turbid. The acetate was then allowed to crystallize out, filtered, and recrystallized several times from alcohol until its melting point was constant at 144°.

Rotation—0.0369 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of $-0.692°$, hence $[\alpha]_D^{19} = -56.26°$.

Mixed with stigmasteryl acetate, m.p. 144° and $[\alpha]_D^{19} = -55.8°$, it did not show a depression of the melting point.

<div align="center">SUMMARY</div>

It has been demonstrated that the oyster, *Ostrea virginica*, contains besides ostreasterol small amounts of stigmasterol, which has been identified as the acetate and tetrabromoacetate.

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THE CONFIGURATIONAL RELATIONSHIP OF α-HYDROXY-n-VALERIC AND α-HYDROXYISOVALERIC ACIDS

By PAUL D. BARTLETT,* MARTIN KUNA, and P. A LEVENE

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Prior to the present investigation the configurations of α-hydroxyisovaleric and α-hydroxyvaleric acids were correlated by indirect methods based on the analogy of their behavior to that of the n-α-hydroxy acids. In the latter case the shift of the direction of rotation on passing from the undissociated acid to the ionized state is invariably dependent on the configuration. It was desired to test the rule with acids containing an isopropyl group. This was particularly important for the reason that in hydrocarbons and in secondary carbinols the isopropyl and the propyl groups, respectively, exercise different effects on the optical rotation.

Inasmuch as the correlation of the configurations of the members of homologous series of secondary carbinols containing a propyl and an isopropyl group had been established, there remained to correlate the configurations of α-hydroxyisovaleric acid to a secondary carbinol containing an isopropyl group. An unsaturated carbinol with the —CH═CH— adjacent to the asymmetric carbon atom served as an intermediary between the two substances. This, on the one hand, was reduced to the saturated secondary carbinol and, on the other, oxidized, through its acetate, to the hydroxy acid. In view of the fact that difficulties were encountered in the resolution of vinylisopropylcarbinol, the crotonylisopropylcarbinol was selected for the work.

The set of correlations is shown in Formulæ I to IX.

Thus 5-methyl-2-hexene-4-ol leads to levo-α-hydroxyisovaleric acid. From Formulæ I to IX it may be seen that the levo un-

* National Research Council Fellow in Chemistry, 1931–32.

$$
\begin{array}{ccccc}
& & & & \text{CH}_3 \\
& & & & | \\
& & & & \text{CH} \\
\text{CH}_3 & \text{COOH} & \text{C}_3\text{H}_7 & \text{CH} \\
| & | & | & | \\
\text{H}\cdots\text{C}\cdots\text{OH} \rightarrow & \text{H}\cdots\text{C}\cdots\text{OH} & \text{H}\cdots\text{C}\cdots\text{OH} \leftarrow & \text{H}\cdots\text{C}\cdots\text{OH} \\
| & | & | & | \\
(n)\text{C}_3\text{H}_7 & \text{C}_3\text{H}_7 & \text{CH} & \text{CH} \\
& & \diagup\diagdown & \diagup\diagdown \\
& & \text{CH}_3 \ \ \text{CH}_3 & \text{CH}_3 \ \ \text{CH}_3 \\
\text{Dextro} & \text{Levo} & \text{Levo} & \text{Levo} \\
\text{I} & \text{II} & \text{III} & \text{IV}
\end{array}
$$

$$
\begin{array}{ccc}
\text{CH}_3 & & \\
| & & \\
\text{CH} & & \\
| & & \\
\ddot{\text{C}}\text{H} \qquad \text{O} & \text{COOH} \qquad \text{O} & \text{COOCH}_3 \qquad \text{O} \\
| \qquad\quad \| & | \qquad\quad \| & | \qquad\qquad \| \\
\text{H}\cdots\text{C}\cdots\text{O}-\text{C}-\text{CH}_3 \rightarrow & \text{H}\cdots\text{C}\cdots\text{O}-\text{C}-\text{CH}_3 \rightarrow & \text{H}\cdots\text{C}\cdots\text{O}-\text{C}-\text{CH}_3 \\
| & | & | \\
\text{CH} & \text{CH} & \text{CH} \\
\diagup\diagdown & \diagup\diagdown & \diagup\diagdown \\
\text{CH}_3 \ \ \text{CH}_3 & \text{CH}_3 \ \ \text{CH}_3 & \text{CH}_3 \ \ \text{CH}_3 \\
\text{Dextro} & \text{Dextro} & \text{Dextro} \\
\text{V} & \text{VI} & \text{VII}
\end{array}
$$

$$
\begin{array}{cc}
\text{COOCH}_3 & \text{COOH} \\
| & | \\
\leftarrow \text{H}\cdots\text{C}\cdots\text{OH} \leftarrow & \text{H}\cdots\text{C}\cdots\text{OH} \\
| & | \\
\text{CH} & \text{CH} \\
\diagup\diagdown & \diagup\diagdown \\
\text{CH}_3 \ \ \text{CH}_3 & \text{CH}_3 \ \ \text{CH}_3 \\
\text{Levo} & \text{Levo} \\
\text{VIII} & \text{IX}
\end{array}
$$
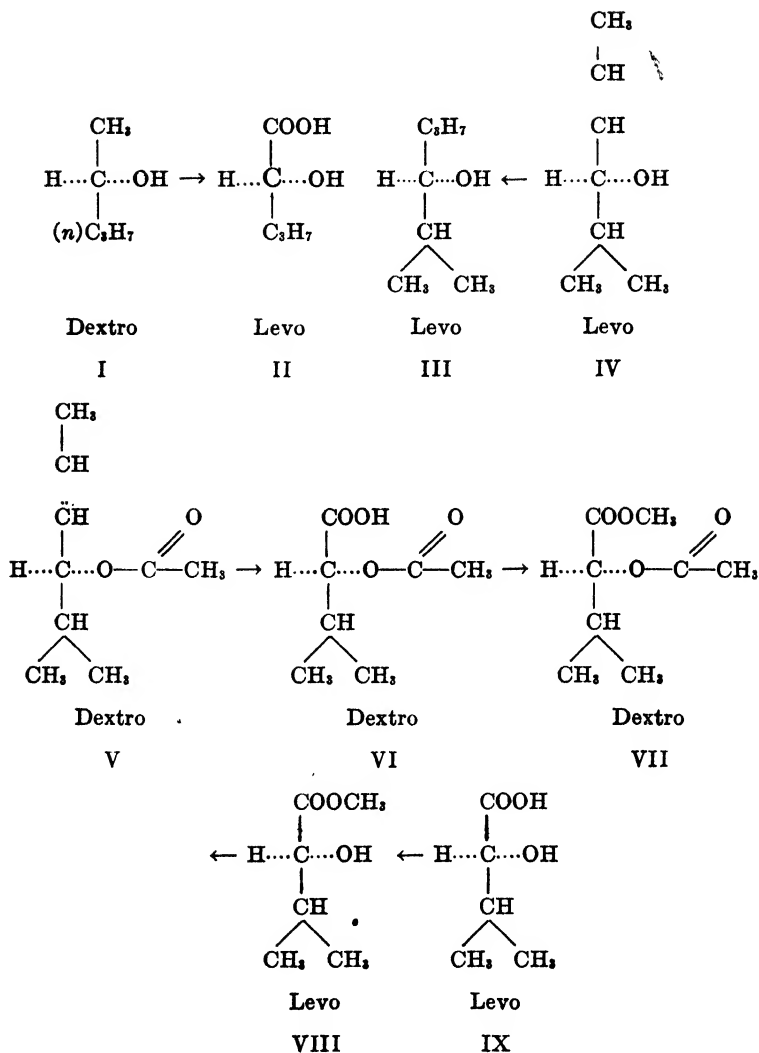
saturated carbinol is configurationally related to both levo-α-hydroxyvaleric and levo-α-hydroxyisovaleric acids.

The same conclusion as to the configuration of the two acids was formerly reached on the basis of the shift in the direction of rotation of the two acids on passing from the undissociated acid to the salt.

5-Methyl-2-Hexene-4-Ol (*Isopropylcrotylcarbinol*)—This substance was prepared, in 54 per cent yield, from crotonaldehyde (Eastman, b.p. 102–103°) and isopropyl bromide (Eastman, purified with concentrated sulfuric acid, then washed with sodium carbonate and water; b.p. 60°) by the Grignard reaction. The product boiled at 51–54° under 15 mm. It was resolved through the acid phthalate strychnine salt in 90 per cent aqueous acetone.

448 gm. of the salt were dissolved in 2200 cc. of 90 per cent acetone and the solution evaporated to 700 cc. The crystals from this were dissolved in 2 liters of solvent and the solution set to crystallize at −10°. The product from this crystallization weighed 93 gm. and a sample of the acid phthalate from it had a specific rotation of

$$[\alpha]_D^{25} = \frac{+0.99° \times 100}{2 \times 2.95} = +16.8° \text{ (in alcohol)}$$

This specific rotation was not further increased by recrystallization. The optically active alcohol was obtained from the acid phthalate by distillation with an excess of 20 per cent sodium hydroxide. The alcohol gave no iodoform test, as would be expected if a partial allyl rearrangement had occurred. It had the following constants: b.p. 51–54° at 15 mm.; $d_4^{25} = 0.8327$.

$$[\alpha]_D^{25} = \frac{+6.50° \times 100}{2 \times 14.66} = +22.2° \text{ (in absolute alcohol)}$$

$$[\alpha]_D^{25} = \frac{+16.12°}{1 \times 0.8327} = +19.36° \text{ (homogenous)}$$

The substance had the following composition.

3.931 mg. substance: 10.600 mg. CO_2 and 4.565 mg. H_2O
$C_7H_{14}O$. Calculated. C 73.61, H 12.37
114.1 Found. " 73.53, " 12.98

Levo-α-hydroxyisovaleraldehyde was prepared by ozonization of dextro-5-methyl-2-hexene-4-ol. 12 gm. of the alcohol (the specific rotation of which had decreased to +13.85° on standing several months) were dissolved in 30 cc. of glacial acetic acid,

surrounded by an ice bath, and ozonized until a sample of the solution no longer decolorized bromine dissolved in carbon tetrachloride. The aldehyde was liberated from the ozonization product with zinc dust in moist ether until the solution no longer colored starch-KI paper. The acetic acid was neutralized with barium hydroxide and sodium carbonate and the ethereal solution dried with sodium sulfate and distilled. The yield of redistilled aldehyde, 3.0 gm., was collected in solid carbon dioxide.

The substance had the following composition.

4.995 mg. substance: 10.500 mg. CO_2 and 4.408 mg. H_2O
$C_5H_{10}O_2$.   Calculated.   C 58.78, H 9.87
102.1   Found.   " 57.32, " 9.87

$$[\alpha]_D^{25} = \frac{-0.24° \times 100}{1 \times 4.48} = -5.4° \text{ (in ether)}$$

Dextro-α-Hydroxyisovaleric Acid—Several attempts were made to prepare this acid from the aldehyde by oxidation, but none of them yielded a satisfactory product.

Dextro-2-methyl-3,4-butanediol was prepared by reduction with sodium amalgam of the levo-aldehyde in aqueous solution. Enough alcohol was added to bring the aldehyde into solution and enough dilute sulfuric acid was added to keep the solution just acid to phenolphthalein. The precipitated salt was filtered off, and the solution extracted seven times with ether. The extract was dried and distilled. The product boiled at 103° at 12 mm.

$$[\alpha]_D^{25} = \frac{+0.19° \times 100}{2 \times 2.46} = +3.9° \text{ (in dry ether)}$$

The substance had the following composition.

4.131 mg. substance: 8.668 mg. CO_2 and 4.220 mg. H_2O
$C_5H_{12}O_2$.   Calculated.   C 57.64, H 11.62
104.1   Found.   " 57.72, " 11.43

An attempt to prepare this glycol by catalytic reduction of the aldehyde with Adams' catalyst, with ferrous sulfate as a promoter, under a pressure of 3 atmospheres resulted in recovery of the unchanged aldehyde.

Dextro-5-methyl-4-hexanol (*propylisopropylcarbinol*) was prepared by hydrogenation of dextro-5-methyl-2-hexene-4-ol (7.33 gm. in 50 cc. of absolute alcohol) under 3 atmospheres pressure, with Adams' catalyst.

The product distilled at 52° at 12 mm. and absorbed no bromine. Its rotation was observed to be +15.03° (homogeneous, 1 dm.). In absolute alcohol it had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+3.08° \times 100}{2 \times 7.68} = +20.1°$$

The substance had the following composition.

4.900 mg. substance: 13.015 mg. CO_2 and 6.015 mg. H_2O

$$\begin{array}{llll} C_7H_{16}O. & \text{Calculated.} & \text{C } 72.33, & \text{H } 13.89 \\ 116.1 & \text{Found.} & \text{`` } 72.43, & \text{`` } 13.82 \end{array}$$

Dextro-4-Acetoxy-5-Methyl-2-Hexene (*Acetate of Isopropylcrotylcarbinol*)—50 gm. of 5-methyl-2-hexene-4-ol

$$[\alpha]_D^{25} = \frac{-9.50°}{1 \times 0.833} = -11.4° \text{ (homogeneous)}$$

were dissolved in 57 gm. of dry pyridine and cooled. Then 57 gm. of acetic anhydride were slowly added. The solution was allowed to stand for 3 days at 15°.

Ether was then added and the solution washed successively with dilute sulfuric acid (until the washings were acid), water, dilute sodium carbonate solution, and finally with water. The extract was dried with drierite. The acetate distilled at 86–87°, $p = 46$ mm. Yield, 40 gm. $d_4^{25} = 0.8789$ (*in vacuo*); $n_D^{25} = 1.4204$.

The substance had the following rotation.

$$[\alpha]_D^{25} = \frac{+18.68°}{1 \times 0.879} = +21.3°; \quad [M]_D^{25} = +33.3° \text{ (homogeneous)}$$

5.405 mg. substance: 3.690 mg. CO_2 and 5.095 mg. H_2O

$$\begin{array}{llll} C_9H_{16}O_2. & \text{Calculated.} & \text{C } 69.17, & \text{H } 10.33 \\ 156.1 & \text{Found.} & \text{`` } 69.07, & \text{`` } 10.54 \end{array}$$

Dextro-α-Acetoxyisovaleric Acid—40 gm. of 4-acetoxy-5-methyl-2-hexene, $[\alpha]_D^{25} = +21.3°$ (homogeneous), were dissolved in 800 cc. of acetone (in four lots), and 200 gm. of powdered potassium

permanganate were added with intermittent heating on the steam bath during 3 days. The color of permanganate persisted after this period.

The precipitate was filtered off and taken up in hot water. The excess permanganate was removed with sodium bisulfite solution and the solution filtered, the manganese dioxide precipitate being washed with hot water a number of times. The combined filtrate and washings were concentrated to a small volume under reduced pressure, and the residue acidified with 50 per cent sulfuric acid and extracted with ether. The extract was washed free of sulfate ions and dried with anhydrous sodium sulfate. The acid distilled at 95–97°, $p = 3$ mm. Yield, 7 gm.

$$[\alpha]_D^{25} = \frac{+1.00° \times 100}{2 \times 5.80} = +8.62°; \quad [M]_D^{25} = +13.8° \text{ (in ether)}$$

The substance had the following composition.

4.020 mg. substance: 7.785 mg. CO_2 and 2.850 mg. H_2O
$C_7H_{12}O_4$.   Calculated.   C 52.47, H 7.56
160.1   Found.   " 52.80, " 7.93

Dextro-α-Acetoxyisovaleric Methyl Ester—2 gm. of α-acetoxyiso-valeric acid, $[\alpha]_D^{25} = +8.62°$ (in ether), were dissolved in 25 cc. of dry ether, cooled, and an excess of diazomethane in ether solution added. This was allowed to stand for 2 days at 15°. The ester was isolated as usual. B.p. 50°, $p = 1$ mm. Yield, 1 gm. $\alpha_D^{25} = +9.25°$ (homogeneous, 1 dm.).

$$[\alpha]_D^{25} = \frac{+0.44° \times 100}{1 \times 4.64} = +9.5°; \quad [M]_D^{25} = +16.5° \text{ (in ether)}$$

The substance had the following composition.

5.000 mg. substance: 10.100 mg. CO_2 and 3.865 mg. H_2O
$C_8H_{14}O_4$.   Calculated.   C 55.14, H 8.10
174.1   Found.   " 55.08, " 8.65

Dextro-α-Hydroxyisovaleric Acid—50 gm. of *d*-valine (Hoffmann-La Roche), $[\alpha]_D^{25} = +27.3°$ (in 20 per cent hydrochloric acid, $c = 5$ per cent), were diazotized according to the procedure

of Fischer and Scheibler.[1] The acid was converted into the sodium salt which was purified by dissolving it in methanol, filtering, and evaporating to dryness.

$$[\alpha]_D^{25} = \frac{-2.20° \times 100}{2 \times 10.0} = -11.0°; \quad [M]_D^{25} = -15.4° \text{ (in water)}$$

This solution was acidified with concentrated hydrochloric acid and then showed

$$[\alpha]_D^{25} = \frac{+0.45° \times 100}{2 \times 8.4} = +2.7°; \quad [M]_D^{25} = +3.16° \text{ (for the free acid)}$$

The sodium salt was dissolved in 50 per cent sulfuric acid and extracted with ether. The extract was dried with drierite and concentrated to a small volume. Pentane was added and the α-hydroxyisovaleric acid crystallized overnight. The crystals were filtered off.

0.502 gm. was dissolved in water to make 15 cc.

$$[\alpha]_D^{25} = \frac{-0.07° \times 100}{4 \times 3.35} = -0.5°; \quad [M]_D^{25} = -0.6° \text{ (for the free acid;}$$
$$c = 3.35\%)$$

This solution was made alkaline with 10 per cent sodium hydroxide and diluted to 20 cc. The rotation then was

$$[\alpha]_D^{25} = \frac{-1.42° \times 100}{4 \times 2.98} = -11.9°; \quad [M]_D^{25} = -16.7° \text{ (for the sodium salt)}$$

1.500 gm. of the acid were dissolved in water to make 15 cc.

$$[\alpha]_D^{25} = \frac{+0.23° \times 100}{4 \times 10.0} = +0.58°; \quad [M]_D^{25} = +0.68° \text{ (for the free acid;}$$
$$c = 10\%)$$

5.098 mg. substance: 9.480 mg. CO_2 and 3.904 mg. H_2O.

	$C_5H_{10}O_3$.	Calculated.	C 50.81, H 8.54
	118.1	Found.	" 50.71, " 8.54

[1] Fischer, E., and Scheibler, H., *Ber. chem. Ges.*, **41**, 2891 (1908).

80 gm. of α-bromoisovaleric acid,[2] $[\alpha]_D^{25} = -6.33°$ (in ether), were converted into the hydroxy acid as described by Fischer and Scheibler.[1] The silver salt was dissolved in 8 liters of hot water and 300 gm. of barium carbonate were added. Hydrogen sulfide was then passed into the mixture. The precipitate was filtered and the filtrate concentrated. The yield of barium salt was 40 gm.

A small portion of the barium salt was converted into the sodium salt with sodium sulfate and the solution showed the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.35° \times 100}{2 \times 5.0} = -3.5°; \quad [M]_D^{25} = -4.9° \text{ (sodium salt in water)}$$

Dextro-α-Hydroxyisovaleric Ethyl Ester—40 gm. of the finely pulverized barium salt of α-hydroxyisovaleric acid, $[\alpha]_D^{25} = -3.5°$ (for the sodium salt), were suspended in 250 cc. of absolute ethanol. Then a solution of 13 cc. of concentrated sulfuric acid in 50 cc. of absolute ethanol was slowly dropped in with shaking.

The mixture was refluxed for 16 hours.

Ether was then added and the excess acid neutralized with powdered anhydrous barium hydroxide. The precipitate was filtered off and the ether and alcohol were distilled from the filtrate at atmospheric pressure. The ester distilled at 112–114°, $p = 110$ mm. Yield, 8 gm.

$$[\alpha]_D^{25} = \frac{+0.30°}{1 \times 1 \text{ (approx.)}} = +0.30°; \quad [M]_D^{25} = +0.40° \text{ (homogeneous)}$$

The substance had the following composition.

3.770 mg. substance: 8.000 mg. CO_2 and 3.302 mg. H_2O

$C_7H_{14}O_3$.	Calculated.	C 57.49, H 9.66
146.1	Found.	" 57.87, " 9.80

Levo-α-Acetoxyisovaleric Ethyl Ester—8 gm. of α-hydroxyisovaleric ethyl ester, $[\alpha]_D^{25} = +0.3°$ (homogeneous), were dissolved in 5 cc. of dry pyridine and cooled. Then 7 gm. of acetic an-

[2] Levene, P. A., Mori, T., and Mikeska, L. A., *J. Biol. Chem.*, **75**, 337 (1927).

hydride were dropped in. The solution was allowed to stand for 16 hours at 15°. The ester was isolated as described for the other acetate. B.p. 80°, $p = 10$ mm.; $d_4^{27} = 1.002$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{-9.85°}{1 \times 1.002} = -9.83°; \quad [M]_D^{27} = -18.5° \text{ (homogeneous)}$$

The substance had the following composition.

4.515 mg. substance: 9.500 mg. CO_2 and 3.495 mg. H_2O

$C_9H_{16}O_4$.   Calculated.   C 57.41, H 8.57

188.1   Found.   " 57.37, " 8.66

THE CONFIGURATIONAL RELATIONSHIP OF α-HXDROXY-n-CAPROIC AND α-HYDROXYISOCAPROIC ACIDS

By PAUL D. BARTLETT,* MARTIN KUNA, and P. A. LEVENE

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

This problem is analogous to that discussed in the preceding communication.[1]   There is, however, an additional point of interest attached to the direction of rotation of the α-hydroxyisovaleric as compared with that of the α-hydroxyisocaproic acid. In the homologous series of hydrocarbons and in the series of secondary carbinols containing an isopropyl group, the direction of rotation of the substances was found to depend on the distance of the isopropyl group from the asymmetric center.   The question is whether a similar state of affairs is observed in the rotations of the two hydroxy acids.

The configuration of α-hydroxyisocaproic acid was established with the acetate of 6-methyl-2-heptene-4-ol as the starting material.

The set of correlations is given by Formulæ I to X.

Thus the set of correlations from Formulæ I to X leads to the conclusion that normal α-hydroxycaproic and α-hydroxyisocaproic acids rotate in opposite directions when they are configurationally related.   It may be recalled that configurationally related α-hydroxyvaleric and α-hydroxyisovaleric acids rotate in the same direction.   Inasmuch as the two normal configurationally related hydroxy acids rotate in the same direction, it follows that configurationally related α-hydroxyisovaleric and α-hydroxyisocaproic acids rotate in opposite directions.   Thus, in the case of α-hydroxy acids the effect of the isopropyl group varies with its distance from the asymmetric carbon atom.

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[1] Bartlett, P. D., Kuna, M., and Levene, P. A., *J. Biol. Chem.*, **118**, 503 (1937).

$$
\begin{array}{cccc}
\text{COOH} & \text{CH}_3 & \text{C}_3\text{H}_7 & \text{C}_3\text{H}_7 \\
| & | & | & | \\
\text{H}\cdots\text{C}\cdots\text{OH} & \cdot\,\text{H}\cdots\text{C}\cdot\cdot\text{OH} \leftarrow & \text{H}\cdots\text{C}\cdots\text{OH} \leftarrow & \text{H}\cdots\text{C}\cdot\text{OH} \\
| & | & | & | \\
(n)\text{C}_4\text{H}_9 & (n)\text{C}_4\text{H}_9 & (n)\text{C}_4\text{H}_9 & (i)\text{C}_4\text{H}_9 \\
\text{Levo} & \text{Dextro} & \text{Dextro} & \text{Dextro} \\
\text{I} & \text{II} & \text{III} & \text{IV}
\end{array}
$$

$$
\begin{array}{ccc}
\text{CH}_3 & \text{CH}_3 & \\
| & | & \\
\text{CH} & \text{CH} & \\
\| & \| & \quad\quad\text{O} \\
\text{CH} & \text{CH} & \quad\quad\|\\
| & | & \quad\text{COOH}\quad\text{O} \\
\text{H}\cdots\text{C}\cdots\text{OH}\cdot & \text{H}\cdots\text{C}\cdots\text{O}\!-\!\text{C}\!-\!\text{CH}_3 & \quad|\quad\quad\| \\
| & | & \text{H}\cdots\text{C}\cdots\text{O}\!-\!\text{C}\!-\!\text{CH}_3 \\
(i)\text{C}_4\text{H}_9 & (i)\text{C}_4\text{H}_9 & \quad|\\
 & & (i)\text{C}_4\text{H}_9 \\
\text{Levo} & \text{Dextro} & \text{Dextro} \\
\text{V} & \text{VI} & \text{VII}
\end{array}
$$

$$
\begin{array}{ccc}
\text{COOCH}_3\quad\text{O} & \text{COOH} & \text{COOC}_2\text{H}_5\quad\text{O} \\
|\quad\quad\| & | & |\quad\quad\| \\
\text{H}\cdots\text{C}\cdots\text{O}\!-\!\text{C}\!-\!\text{CH}_3 & \text{H}\cdots\text{C}\cdots\text{OH}\rightarrow & \text{H}\cdots\text{C}\cdots\text{O}\!-\!\text{C}\!-\!\text{CH}_3 \\
| & | & | \\
(i)\text{C}_4\text{H}_9\cdot & (i)\text{C}_4\text{H}_9 & (i)\text{C}_4\text{H}_9 \\
\text{Dextro} & \text{Dextro} & \text{Dextro} \\
\text{VIII} & \text{IX} & \text{X}
\end{array}
$$
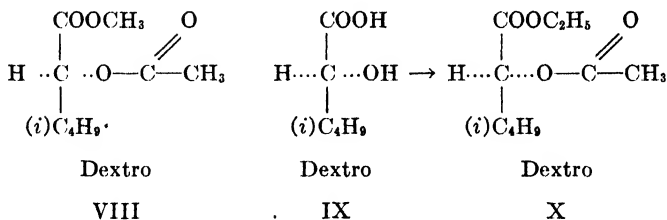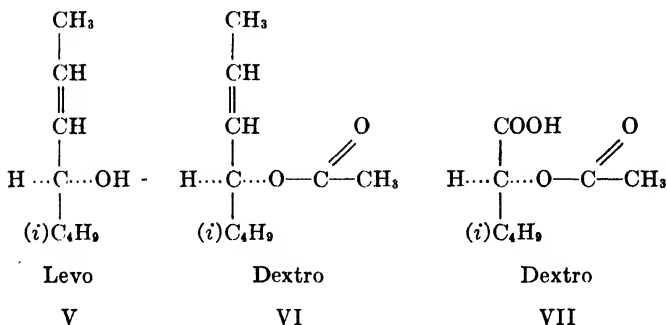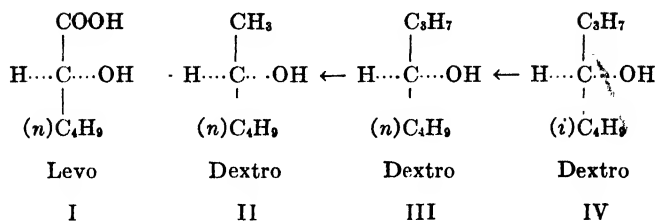
It may further be mentioned that in the configurationally related α-hydroxyisovaleric and α-hydroxyisocaproic as well as in the normal α-hydroxy acids, the shift of rotation on passing from the undissociated acid to the ionized state is in the same direction. Thus it is justified to determine the configurations of aliphatic α-hydroxy acids by observing the direction of the shift of rotation on passing from the undissociated acid to the ionized form. Those in which the shift is to the right belong to the *d* series.

EXPERIMENTAL

5-Methyl-1-hexene-3-ol (*vinylisobutylcarbinol*) was prepared from acrolein and isobutylmagnesium bromide in 35 per cent yield.

The acid phthalate was made in pyridine by the usual procedure and purified through the sodium salt. This phthalate could not be resolved with either brucine or strychnine.

After four recrystallizations of the brucine salt in acetone, 145 gm. of an original 391 gm. yielded an acid phthalate having zero rotation. A further fractionation left 90 gm. of salt. The phthalate from this was still inactive. There was an apparent transient rotation but this was always small.

6-Methyl-2-heptene-4-ol (*isobutylcrotylcarbinol*) was prepared in 50 per cent yield from crotonaldehyde and isobutylmagnesium bromide. It boiled at 69° at 12 mm. Resolution was effected through the strychnine salt of the acid phthalate which was recrystallized from 90 per cent acetone. The resolution was not carried to the maximum, since a good quantity of active material was obtained by decomposition of the syrup which remained after all the possible crystalline material had been removed from the concentrated mother liquors containing the strychnine salt. The correlations were carried out with the alcohol so obtained. It had a rotation (homogeneous) of $-1.22°$ in a 1 dm. tube. This, combined with the density of 0.835 given by von Auwers and Westermann[2] means a specific rotation of $-1.46°$ for this material.

It was noticed that the acid phthalate of this carbinol, on heating, gives a good yield of a mobile unsaturated liquid boiling at 30° at 12 mm., with an odor suggesting that of oil of wintergreen, probably 6-methyl-2,4-heptadiene.

Dextro-6-methyl-4-heptanol (*propylisobutylcarbinol*) was prepared by hydrogenation of levo-6-methyl-2-heptene-4-ol under pressure with Adams' catalyst. The reduction of 20 gm. in absolute alcohol required a little over an hour. The product boiled at 72° at 15 mm., and gave a rotation of $+3.00°$ (homogeneous) in a 1 dm. tube.

Dextro-α-Hydroxyisocaproic Aldehyde—Levo-6-methyl-2-heptene-4-ol was ozonized in glacial acetic acid and the product isolated following the conditions given by Helferich.[3] The fraction boiling at 79° at 18 mm. was collected.

[2] von Auwers, K., and Westermann, H., *Ber. chem. Ges.*, **54**, 2993 (1921).

[3] Helferich, B., *Ber. chem. Ges.*, **52**, 1128 (1919).

The material was undoubtedly contaminated with a little acetaldehyde.

$$[\alpha]_D^{25} = \frac{+0.58° \times 100}{2 \times 4.81} = +6.0° \text{ (in absolute alcohol)}$$

3.284 mg. substance: 7.030 mg. CO_2 and 2.870 mg. H_2O

$\quad\quad C_6H_{12}O_3$. Calculated. C 62.03, H 10.40

$\quad\quad$ 116.1 $\quad$ Found. $\quad\quad$ " 58.37, " 9.78

Dextro-4-Acetoxy-6-Methyl-2-Heptene (*Acetate of Isobutylcrotylcarbinol*)—36 gm. of 6-methyl-2-heptene-4-ol

$$[\alpha]_D^{25} = \frac{-0.76°}{1 \times 0.835} = -0.91° \text{ (homogeneous)}$$

were dissolved in 40 gm. of dry pyridine, cooled, and 40 gm. of acetic anhydride were slowly added. The solution was allowed to stand at a temperature of 15° for 3 days. The acetate was isolated in the usual way. Yield, 36 gm. B.p. 88–90°, $p = 30$ mm.; $d_4^{25} = 0.8696$ (*in vacuo*); $n_D^{25} = 1.4249$.

$$[\alpha]_D^{25} = \frac{+11.9°}{1 \times 0.870} = +13.7°; \quad [M]_D^{25} = +23.3° \text{ (homogeneous)}$$

4.492 mg. substance: 11.595 mg. CO_2 and 4.280 mg. H_2O

$\quad\quad C_{10}H_{18}O_2$. Calculated. C 70.53, H 10.66

$\quad\quad$ 170.1 $\quad$ Found. $\quad\quad$ " 70.39, " 10.66

Dextro-α-Acetoxyisocaproic Acid—36 gm. of 4-acetoxy-6-methyl-2-heptene, $[\alpha]_D^{25} = +13.7°$ (homogeneous), were oxidized with powdered potassium permanganate as described for the 5-methyl-2-hexene-4-ol.[1] The acid distilled at 127°, $p = 5$ mm. Yield, 6.5 gm.

$$\alpha_D^{25} = +11.74° \text{ (homogeneous, 1 dm.)}$$

$$[\alpha]_D^{25} = \frac{+2.28° \times 100}{2 \times 11.5} = +9.91°; \quad [M]_D^{25} = +17.3° \text{ (in ether)}$$

0.1160 gm. substance: 6.815 cc. 0.1 N NaOH

$\quad\quad C_8H_{14}O_4$ (174.1). Found, mol. wt. = 170.2

Dextro-α-Acetoxyisocaproic Methyl Ester—5 gm. of *α*-acetoxy-isocaproic acid, $[\alpha]_D^{25} = +9.91°$ (in ether), were treated with an ethereal solution of diazomethane as usual. The ester distilled at 68°, $p = 5$ mm. Yield, 4 gm. $d_4^{27} = 1.020$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{+10.7°}{1 \times 1.02} = +10.5°; \quad [M]_D^{27} = +19.8° \text{ (homogeneous)}$$

3.099 mg. substance: 6.506 mg. CO_2 and 2.385 mg. H_2O
$C_9H_{16}O_4$. Calculated. C 57.41, H 8.57
188.1   Found.   " 57.25, " 8.61

Levo-α-Hydroxyisocaproic Acid—40 gm. of *l*-leucine (Kahl-baum), $[\alpha]_D^{25} = +20°$ (in 20 per cent hydrochloric acid), were diazotized according to the procedure of Scheibler and Wheeler.[4] The acid was converted into the barium salt. Yield, 20 gm.

$$[\alpha]_D^{25} = \frac{-1.18° \times 100}{2 \times 5.0} = -11.8°; \quad [M]_D^{25} = -23.6° \text{ (barium salt in water)}$$

Levo-α-Hydroxyisocaproic Ethyl Ester—20 gm. of the barium salt of α-hydroxyisocaproic acid, $[\alpha]_D^{25} = -11.8°$ (in water), were finely pulverized and suspended in 100 cc. of absolute ·ethanol. Then 6 cc. of concentrated sulfuric acid in 25 cc. of absolute ethanol were slowly added with shaking. This was refluxed for 16 hours. Ether was added and the solution shaken with concentrated calcium chloride solution. The extract was dried with sodium sulfate. The ester distilled at 118°, $p = 90$ mm. Yield, 10 gm.

$$[\alpha]_D^{25} = \frac{-6.85°}{1 \times 0.97} = -7.06°; \quad [M]_D^{25} = -11.3° \text{ (homogeneous)}$$

2.805 mg. substance: 6.200 mg. CO_2 and 2.590 mg. H_2O
$C_8H_{16}O_3$. Calculated. C 59.95, H 10.07
160.1   Found.   " 60.27, " 10.33

Levo-α-Acetoxyisocaproic Ethyl Ester—5 gm. of α-hydroxy-isocaproic ethyl ester, $[\alpha]_D^{25} = -7.06°$ (homogeneous), were dis-solved in 4 gm. of pyridine, cooled, and 5 gm. of acetic anhydride were added. This was allowed to stand at a temperature of 15° for 36 hours. The substance was isolated as usual. B.p. 74°, $p = 1$ mm. Yield, 3 gm. $d_4^{27} = 0.989$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{-34.4°}{1 \times 0.989} = -34.8°; \quad [M]_D^{27} = -70.3° \text{ (homogeneous)}$$

4.292 mg. substance: 9.300 mg. CO_2 and 3.410 mg. H_2O
$C_{10}H_{18}O_4$. Calculated. C 59.36, H 8.97
202.1   Found.   " 59.08, " 8.89

[4] Scheibler, H., and Wheeler, A. S., *Ber. chem. Ges.*, **44**, 2684 (1911).

STUDIES ON PORPHYRIA*

I. OBSERVATIONS ON THE FOX-SQUIRREL, SCIURUS NIGER

By WILLIAM J. TURNER

(From Wilkinsburg, Pennsylvania)

In the past few years there has been a wide development of interest in porphyrins and porphyria. A number of new cases of ·porphyria have been studied both in this country and abroad (1–6). The problems presented by the chemical studies on these cases lie in a new and fascinating field of pyrrole metabolism. Since there is no suitable introduction to this subject in English, it seems fitting that a brief statement of our present status regarding this matter should be made. For more detailed information the reader is referred to other sources (7–10).

We may consider that the building blocks of porphyrins are disubstituted pyrroles, one of the substituents being always a methyl group.
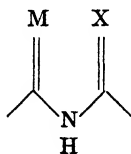
If four methylethyl pyrroles be joined by formyl bridges, an etioporphyrin is formed. Of the four possible isomers derivatives of only etioporphyrins I and III have been found in nature (8). In the

formulæ presented for etioporphyrins M and E stand for methyl and ethyl groups respectively.
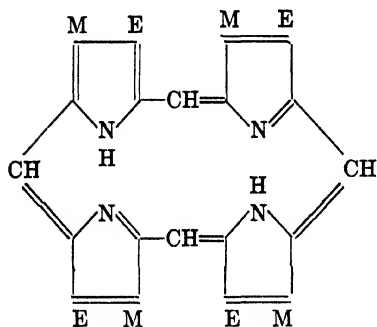
Etioporphyrin I
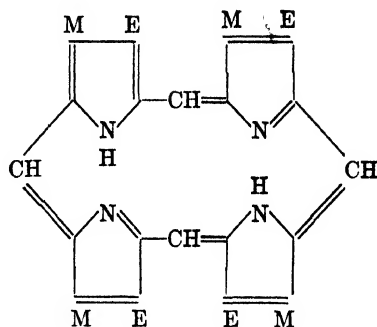
Etioporphyrin III

For example, of the four possible tetramethyl, tetrapropionic acid *porphins*, known as coproporphyrins, only isomers (I) and (III) have been found (8).
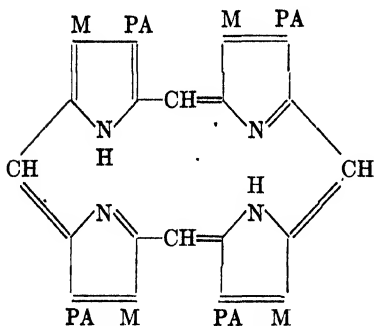
Coproporphyrin I

(PA = CH_2CH_2COOH)
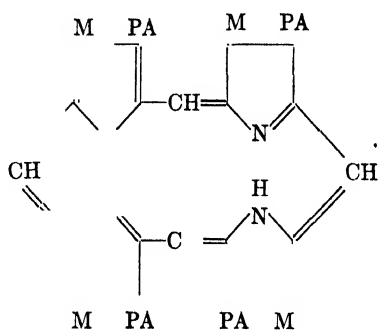
Coproporphyrin III

The same is true of the uroporphyrins. The exact constitution of these octacarboxylic acid porphyrins is not yet proved by synthesis, but the most likely formulæ for the isomers so far found are shown in the accompanying diagrams (11).
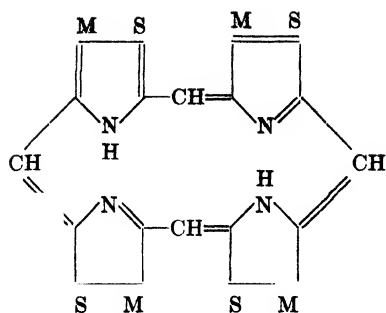
Uroporphyrin I

(S = CH(COOH)CH₂COOH)
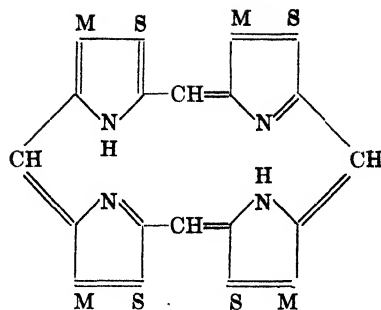
Uroporphyrin III

Probably the most important porphyrin in nature is proto-porphyrin IX (8). There are fifteen possible isomers of this
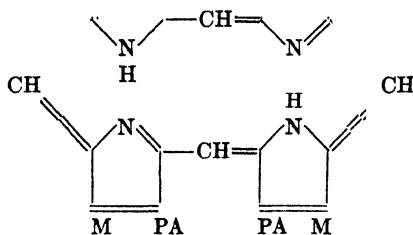
Protoporphyrin IX

compound, but this one, homologous with etioporphyrin III, is the only one so far demonstrated in nature. The importance of it lies in the fact that its iron complex, bound to various proteins and in different physical states, is recognized as hemoglobin, myoglobin, catalase, and probably one of the components of cyto-chrome (12–15). Thus it is the basis of the most important respiratory pigments in both animal and plant life. Furthermore, every other respiratory pigment of a hemin nature so far dis-covered has a porphyrin of Type III as its nucleus.

Now the very existence of porphyrins of these two types bespeaks a dual method of porphyrin synthesis, as Fischer (16) first pointed out, and as Borst and Königsdorfer have demonstrated (2).   Fischer has pointed out (7, 8) that a porphyrin of one type cannot give rise to a porphyrin of another type without complete destruction not only of the porphyrin ring but also of the tetrapyrrole structure.

We can now see more clearly some of the problems before us. We have not only to learn whence pyrroles arise, but also which ones are produced.   The intermediary pyrrole metabolism must be studied to determine if possible by what steps proto-, copro-, and uroporphyrin are formed.   Finally we must determine what factors make for the normally great excess of Type III porphyrin formation over Type I, and under what conditions this relationship is disturbed.

Undoubtedly the study of such disturbed metabolism will prove of great value in determining the answers to the problems set forth, but such study is beset with many difficulties.   One of the greatest of these is that, up to the present, we have had to rely upon chance encounters with sick patients, who either die quickly or escape from observation on improving.   The rare slaughter-house animal with so called animal ochronosis, whose bones are colored red with uroporphyrin I (17), is of little further use in the study of pyrrole metabolism.   Some rabbits chronically poisoned with sulfonal excrete uroporphyrin (18), and these may profitably be studied; yet such animals lead a precarious existence and the induction of porphyria in them is by no means easy or constant.

Other possible experimental animals are not only generally inaccessible but also inherently difficult to study: *Pteria vulgaris*, a mussel from the Persian Gulf, in the shells of which Fischer and Haarer (19) found uroporphyrin I; birds of the genus *Turacus* from Africa, in whose wing feathers the copper complex of uroporphyrin I is found (20); *Asio flammeus*, a bird of sub-Arctic habitat, in whose bones Derrien and Turchini (21) found a porphyrin which is probably uroporphyrin; and finally mammalian fetuses in whose bones and blood stream uro- and coproporphyrin have been found (2, 22, 23); none of these fulfils the requirements of an experimental animal to any degree of satisfaction.

It is therefore encouraging to find that the fox-squirrel, a common and widely scattered denizen of the American forests, has a physiological porphyria. This animal, which matures in about a year and reaches a weight of 600 to 900 gm., easily adapts itself to cage life and becomes quite tame.

Although the farmers of Pennsylvania have long known that the bones of the fox-squirrel are red, it has entirely escaped scientific observation. Since I first discovered this phenomenon about a year ago, I have examined the bones of three recently killed fox-squirrels and have found the same condition in each. This red color turns out to be due to the presence of uroporphyrin I.

In an effort to find other animals similarly affected I turned to Mr. J. K. Doutt, Curator of Mammals of the Carnegie Museum, Pittsburgh, for whose cooperation and suggestions I am grateful. He opened to me the bone collection under his care, consisting of more or less complete skeletons of thousands of small mammals. He also aided me in obtaining from each of the larger museums in the country some notes on pink or red or brown bones in their collections. These covered not only mammalian but also amphibian and reptile bones. As a result an enormous number of bones were examined in this study.

The results of this may be briefly summarized. A few mammals other than rodents have brownish bones; nothing of this sort was found in any lower vertebrates examined, although the birds were not studied very thoroughly. Among mammals only in the Sciuridæ do really red bones appear to occur, though many other rodents have pinkish bones. I have found in *Tamias striatus* pink bones and pink urine, both apparently due to the presence of porphyrin. No other animal studied, however, has had the marked porphyria exhibited by *Sciurus niger*.

EXPERIMENTAL

Bones—It was found on dissecting freshly killed adult fox-squirrels that the diaphyses of the long bones were yellowish. The metaphyses and the epiphyses, particularly those which are the last to unite, had a pink to a dark red hue. The smaller bones were almost uniformly pink to red. On standing in the air the yellow diaphyses assumed a more and more intense red color, finally coming to look like the epiphyses. In ultraviolet light

the fresh bones shone with a bright red fluorescence; when older, the fluorescence was a darker, duller red.   The teeth, although pink at the gum margin, did not fluoresce, nor did the red-brown anterior surfaces of the incisors.

Bone Marrow—The bone marrow was generally red, only a small proportion having a fatty, yellow appearance.   Fresh and alcohol-fixed smears stained with Jenner's stain showed, in addition to many leucoblastic cells and erythrocytes of the normocytic series, a number of large cells with a pale green-staining cytoplasm and dense chromatin which appeared to be megaloblasts.

Unstained smears were studied by fluorescence microscopy. Since this is a technique rarely employed in this country, a short description of it follows.

The principle of fluorescence microscopy is that an unstained, thin section of tissue, examined in ultraviolet light, will be visible by reason of the fluorescence of compounds in the tissue.   The intensity and color of the emitted light will provide a clue as to the nature and concentration of certain substances in the tissue, which clue can be followed up by various methods of chemical microscopy.   Fats, in general, fluoresce yellowish; proteins emit a blue light.   Porphyrins shine red in ultraviolet.   This is a phenomenon which is extremely rare, and when seen is presumptive evidence for the presence of porphyrins.   Only in the liver and bile passages is there much reason to suspect that other compounds may be the source of red fluorescence (24, 25).

. The methods of fluorescence microscopy may be exceedingly complex (2, 26), but for the present purpose a very simple method was employed.   As a source of ultraviolet light either sunlight or the light from a 5 ampere arc, with therapeutic C carbons, was used.   This was passed, by a reflector, through Corning glass filters No. 986 and No. 348 which replaced the substage condenser of a Zeiss monocular microscope.   The tissue to be examined was placed on a thin cover-slip rather than on a slide.   Under these conditions the only visible light impinging on the cover-slip had wave-lengths below 4300 Å., and no visible red could be detected with a spectroscope.

In examining the smears of bone marrow, after the light on the cover-slip was adjusted, a blanket was thrown over my head as a hood, and after several minutes adjustment to the darkness observations were made.

Under these conditions a thin sliver of rib shone bright pink. Unstained smears of bone marrow had considerable yellow-fluorescing fat globules, and here and there clumps of cells which had a dull red fluorescence. On removing the light filters and staining the smear on the microscope stage, the fluorescent cells were found to be megaloblasts.

Extraction of Porphyrin from Bone—From the second squirrel examined there had been obtained, by Fischer's method of extracting porphyrin from bone (9), some 0.29 mg. of uroporphyrin from 17 gm. of dried and defatted bone. This was sent to Professor Fischer, who identified it spectroscopically as uroporphyrin, but found it mixed with fat and of such small amount as to preclude a melting point determination of the ester.

The method given below gave a much better yield of a purer uroporphyrin than Fischer's method. It is a modification of a technique used by Fink and Fikentscher (17).

After the long bones of a freshly killed adult squirrel were cleaned, they were covered with 5 per cent HCl for 48 hours and then evaporated to dryness *in vacuo*. The soft remnants of bones were cut fine with scissors and returned to the pink bone powder. 200 cc. of 5 per cent sulfuric acid-methyl alcohol were allowed to stand over the bone powder for 1 week, filtered off, and replaced by a similar quantity of acid alcohol. After the second extraction only a very faint pink remained in the bone, which was not removed by a third extraction.

The acid alcohol extracts were combined, added to 25 cc. of chloroform in a separatory funnel, and cold distilled water added slowly to make a liter. The chloroform soon settled out red and turbid. After being washed thrice with 1 N HCl,[1] the extract was filtered through Whatman No. 5 filter paper and evaporated to dryness *in vacuo*. In order to separate the porphyrin ester from fats the residue was taken into ether, from which the porphyrin was removed by repeated extraction with 7 to 8 per cent HCl.

This acid solution was next evaporated to dryness *in vacuo*, and the residue covered with a few drops of glacial acetic acid and concentrated HCl. After a few days in the cold saponification was complete. Dilution and adjustment to pH 4.0 were followed

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[1] Previous experience had shown that if the chloroform solution were washed with water or alkali, the consequent precipitation of calcium salts diminished the yield of porphyrin.

by ether extraction. No ether-soluble porphyrin was found. On standing the uroporphyrin precipitated from the aqueous solution, leaving a pale yellow, porphyrin-free supernatant fluid. Repre-cipitation left the supernatant fluid colorless.

The porphyrin was now esterified in acid alcohol again, taken into chloroform, washed with sodium bicarbonate and then with water, dried with anhydrous sodium sulfate, filtered, and evap-orated to dryness *in vacuo*. The residue was washed with petro-leum ether and then with cold carbon tetrachloride, taken into a small volume of chloroform, and crystallized by addition of boiling methyl alcohol and standing. After the third recrystallization the melting point was found to be 291° (uncorrected). In chloro-form the maxima of the absorption spectra were I 627, II 571, III 535, IV 500 $m\mu$. These agree with the published figures for uroporphyrin (10). Superimposing a spectrum of uroporphyrin ester from Fischer by use of a comparison spectroscope gave com-plete agreement of the spectra. Therefore the bone porphyrin is uroporphyrin I.

Urine—At times the urine of the fox-squirrel was a bright, clear sherry-red on being passed. It then had an absorption spectrum as follows:

I 580–552 $m\mu$; II 545–518 $m\mu$; end-absorption 515 $m\mu$ (I, II)
567 . 530

This was not changed by addition of sodium hyposulfite and potassium cyanide in alkaline solution. The urine became a little darker on addition of concentrated HCl, and the bands shifted at once to

I 602–578 $m\mu$; II 560–547 $m\mu$; end-absorption 510 $m\mu$
591 555

With this the green fluorescence of the native urine changed to red. On standing any length of time the acid spectrum of uro-porphyrin appeared: I 591 $m\mu$; II 576 $m\mu$; shadow to III 553 $m\mu$.

At other times the urine was passed a light yellow color, darken-ing in the light and air. Neither the yellow nor the red urine contained albumin or sugar, nor gave Ehrlich's aldehyde or Schlesinger's zinc acetate reactions for bile pigments. There was no evidence for the presence of urofuscin, the dark red pigment so often prominent in the urine in human porphyria.

On extracting the urine with ether, after acidification with acetic acid, a red pigment went into the ether. Washing with distilled water followed by extraction with 5 per cent HCl brought out a minute amount of coproporphyrin spectroscopically identifiable, but the greater amount of the pigment remained in the ether. Here it gave a blue fluorescence and absorption maxima at 561 and 525 $m\mu$. 30 per cent HCl did not remove it from ether, but 0.1 N NaOH brought it out quantitatively. In this solvent its absorption maxima were I 562 to 561, II 526 to 525 $m\mu$ (I, II). It now had a bright green fluorescence in ultraviolet light. On standing in strong aqueous acid the spectrum changed to that of uroporphyrin.

The absorption spectrum of this pigment is that of a metal complex, apparently of uroporphyrin. The amount obtained did not permit of absolute identification of the porphyrin, and so far I have not been able to identify the metal.

The metal complex precipitated from its aqueous solution at pH 4.4. It was then found that in 2 per cent sulfuric acid-methyl alcohol esterification took place with little breakdown to uroporphyrin. This uroporphyrin went into solution in the acid alcohol, the metal complex remaining practically insoluble. Filtration and washing with acid alcohol left the complex ester on the filter paper, from which it was dissolved in chloroform. This was washed and dried in the usual way and crystallized from $CHCl_3$-MeOH. The crystals were washed with petroleum ether and cold carbon tetrachloride; in the latter they were slightly soluble. After three recrystallizations the melting point was 284–288°; after a fourth it was 291°, sharp (uncorrected). Attention is to be called to the fact that this is the melting point of the uroporphyrin obtained from the bone.

The metal complex ester crystallized in long, thin hairs, coppery red and dichroic, which grossly and microscopically greatly resemble those of uroporphyrin I ester.

After ether extraction of the urine the residue was passed through Merck's aluminum oxide, c.p., according to the method of Waldenstrom (27). In the ammonium hydroxide elution uroporphyrin came out. The precipitate obtained by addition of acetic acid was esterified and purified in the usual way. The amount was too small for melting point determination, but the crystals were typical of uroporphyrin. The absorption spectrum,

however, varied slightly from that of pure uroporphyrin. In $CHCl_3$, I 626, II 569, III 533, IV 500 $m\mu$. The maxima for II and III lie slightly to the blue of the published figures, probably owing to the presence of small amounts of metal complex.

Note must be made of the volume of urine with which this work was done. A single squirrel was furnished me by the Pennsylvania Board of Game Commissioners, through the courtesy of Mr. Richard Gerstell, Chief of the Division of Game Research and Distribution. This animal was placed in a large bird cage, where it stayed a week before cutting through a wire with its teeth and escaping.

During this week urine and feces were collected. It was necessary to visit the cage frequently and when the squirrel urinated to remove the bottom of the cage, pick out, and discard the food débris, separate and save the feces, and then wash off the urine into a dark bottle under petroleum ether. This made it impossible to measure the exact quantity of urine passed, but it was estimated that less than 20 cc. were obtained for study. The extent of the animal's disturbed porphyrin metabolism may then be judged from the fact that from this urine it was possible to identify coproporphyrin spectroscopically, obtain crystals of uroporphyrin ester, and determine the melting point of the metal complex of uroporphyrin ester.

Feces—15 gm. of dried feces were obtained and worked up by Fischer's method (9) for coproporphyrin. On removing the porphyrins from the first ether extraction by 5 per cent HCl, a marked paucity of copromesobiliviolin as against the amount present in human feces was noted. This HCl extract was partly neutralized with $NaHCO_3$ and the chloroform-soluble porphyrins removed. These, in chloroform, had the following absorption maxima: I 631, II 575, III 540, IV 505 $m\mu$. The small amount of porphyrin present did not permit purification, but presumably the porphyrin was mostly protoporphyrin.

Purification of the coproporphyrin remaining in the HCl was conducted in the usual fashion, with ether extraction and removal therefrom by 0.1 per cent HCl. For measurement of the absorption spectrum, the coproporphyrin had to be taken into ether again and removed by 10 per cent HCl, in which the absorption maxima were I 592 to 591, II 548 to 547, III indefinite shadow about 494 to 490 $m\mu$. In 0.1 N NaOH the spectrum was I 617,

II 563, III 536, IV 502 $m\mu$.   The amount of coproporphyrin found in 15 gm. of feces corresponds roughly with the amount found normally in human feces, on a meat-free diet.   Crystals of the ester could not be obtained.

DISCUSSION

It has been said above that fetal mammals have red bones (2, 22).   This is particularly true of rodents, and the bones of rats at birth are often quite red.   Borst and Königsdorfer have shown that this deposition of uroporphyrin in fetal bones is associated with the fetal megaloblastic type of erythropoiesis, and since their work it is recognized that megaloblasts are the probable site of elaboration of uroporphyrin (2, 28).

It would seem that in the rat, rabbit, and guinea pig the elaboration of uroporphyrin ceases toward the end of fetal life, and the growing bones quickly lose their red appearance.   If we may judge from the fact that rabbits may develop sulfonal porphyria, which dogs and cats do not do, we may suppose that in rodents it may be possible to induce a return to a fetal stage, not only of erythropoiesis but also of pyrrole metabolism.

Now in the fox-squirrel the evidence educed above points to the conclusion that this animal persists in its fetal mechanism of pyrrole metabolism, with persistent megaloblastic development into adult life.   It appears, too, that in all probability *Sciurus niger* is not alone in this regard, but that, scattered throughout the rodents this phenomenon crops up with greater or lesser duration and degree with little regard for phylogenetic relations.

This matter of persistence of fetal medullary activity draws porphyria into close connection with pernicious anemia.   Yet that there is some deep, fundamental difference between them is obvious, and is further accentuated by the fact that, although there is an increased porphyrin excretion in pernicious anemia, it is not uroporphyrin.   There may be the same apparent return to fetal erythropoiesis, but in one case there is a superimposed disturbance of pyrrole metabolism.

The present studies on squirrels are to be continued.   Studies of the hematology of various Sciuridæ will be reported in their proper place later.

SUMMARY

A brief review of the present status of our knowledge of porphyrins has been given, together with leading references to the literature.

Studies on *Sciurus niger* demonstrate that it is a mammal which has a physiological porphyria extending into adult life. This is a porphyria with elaboration of Type I porphyrins.

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COLORIMETRIC DETERMINATION OF THE COMPONENTS OF 3,4-DIHYDROXYPHENYLALANINE-TYROSINE MIXTURES

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It has proved necessary in recent investigations concerned with a possible mechanism for melanin formation to determine quantitatively the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. The method suggested by Schmalfuss and Lindemann (9) is based upon the previous observation of Schmalfuss and Werner (10) that the tyrosinase from the hemolymph of *Arctia caja* catalyzes the conversion of 3,4-dihydroxyphenylalanine and tyrosine to melanin, this conversion being much more rapid in the case of 3,4-dihydroxyphenylalanine than in the case of tyrosine. The melanin thus produced is determined colorimetrically.

The scheme presented in this paper is based on simple chemical procedures, no enzyme being required.

Determination of 3,4-Dihydroxyphenylalanine

Reagents—

1. 0.5 N hydrochloric acid.

2. Nitrite-molybdate reagent. Dissolve 10 gm. of sodium nitrite and 10 gm. of sodium molybdate in 100 cc. of distilled water.

3. 1 N sodium hydroxide.

4. Standard solution. Dissolve 50 mg. of pure 3,4-dihydroxyphenylalanine in 500 cc. of distilled water contained in a liter volumetric flask. Add 2 cc. of 0.1 N hydrochloric acid and enough distilled water to make a volume of 1 liter. Preserve under toluene.

5. Alternative standard. If 3,4-dihydroxyphenylalanine is not available, catechol can be used as a standard. Dissolve 192 mg. of catechol in enough distilled water to make a volume of 1 liter Preserve under toluene. Dilute 10 cc. of this stock solution to a volume of 100 cc. to make a standard solution. A green Wratten No. 61 filter (supplied by the Eastman Kodak Company) must be used in making the reading if this standard is used. 1 cc. of the catechol standard is equivalent to 1 cc. of the 3,4-dihydroxyphenylalanine standard described above.

Procedure—Place 1 cc. of unknown solution (containing 0.02 to 1.0 mg. of 3,4-dihydroxyphenylalanine) in a test-tube graduated at 5 cc. Place 1 cc. of standard solution in a similar test-tube. Add to each test-tube, in the order given, the following reagents, mixing well after each addition: 1 cc. of 0.5 N hydro-

TABLE I

Determination of 3,4-Dihydroxyphenylalanine

Theoretical concentration	Determined concentration	Theoretical concentration	Determined concentration
mg. per l.	*mg. per l.*	*mg. per l.*	*mg. per l.*
20.0	20.5	60.0	60.8
30.0	30.5	70.0	69.2
40.0	40.1	80.0	79.4
50.0	50.4	100.0	100.2

chloric acid, 1 cc. of nitrite-molybdate reagent (a yellow color results at this point), 1 cc. of 1 N sodium hydroxide (a red color results), and enough distilled water to make a volume of 5 cc. Compare in a Duboscq colorimeter. The green Wratten No. 61 filter must be used if the catechol standard is used; it may be used with the 3,4-dihydroxyphenylalanine standard if the analyst has difficulty in matching red colors. Tyrosine does not interfere with this determination.

The accuracy of this method is indicated by Table I, which lists results obtained by the analysis of pure 3,4-dihydroxyphenylalanine solutions.

Discussion—The determination of 3,4-dihydroxyphenylalanine is based upon the fact that this substance gives a yellow color with nitrous acid, the yellow color changing to an intense orangered in the presence of excess sodium hydroxide. Castiglioni (1)

has observed that compounds containing phenolic OH groups yield colored compounds when heated with sodium nitrite. He believes that hydrogen ions from the phenolic OH groups unite with nitrite ions from the sodium nitrite, the molecular nitrous acid then forming NO compounds with the phenols. Various
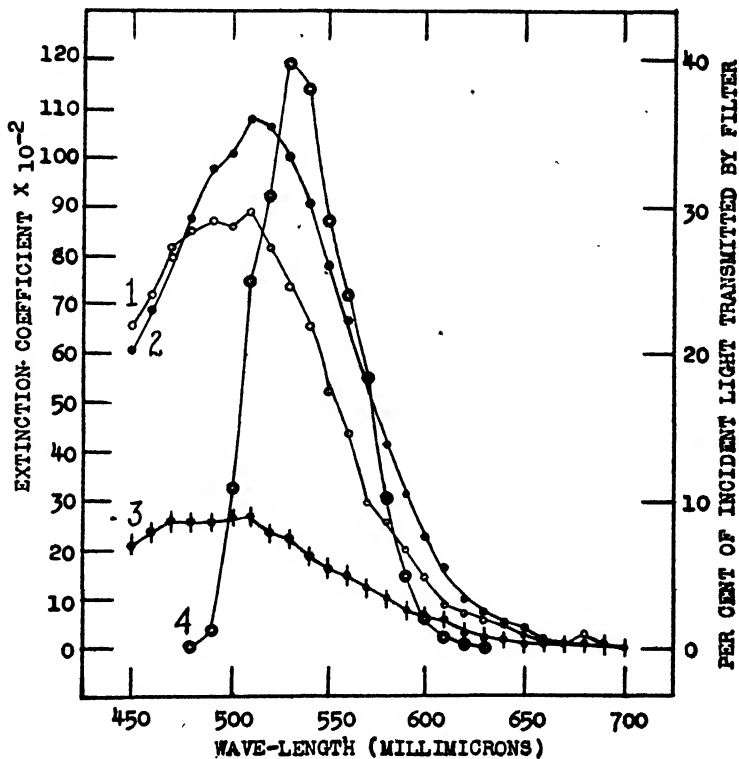
Fig. 1. Absorption spectra for colored solutions and transmission curve for green Wratten No. 61 filter. Curve 1, 3,4-dihydroxyphenylalanine derivative; Curve 2, catechol derivative; Curve 3, tyrosine derivative; Curve 4, Wratten filter.

investigators (6, 8) have used nitrites to detect the presence of epinephrine.

Purpose of the Sodium Molybdate—If sodium nitrite is added to an acid solution of 3,4-dihydroxyphenylalanine, the nitrous acid which is formed decomposes fairly rapidly, and the final intensity

of color which is produced depends to some extent on the time during which the reaction is allowed to proceed. The sodium molybdate prevents the rapid decomposition of the nitrous acid. In addition, it causes an increase in color production of about 50 per cent in the case of 3,4-dihydroxyphenylalanine, and of about 15 per cent in the case of catechol.

Purpose of Acidifying Standard Solution—Alkaline, neutral, or even very slightly acid solutions of 3,4-dihydroxyphenylalanine rapidly turn red, later depositing a precipitate of melanin. The addition of small amounts of hydrochloric acid prevents this change.

TABLE II

Colors Produced by Various Compounds

Compound	Color after addition of HCl and nitrite-molybdate	Color after addition of NaOH
Ephedrine.......................	Colorless	Colorless
Phenol...........................	Light yellow (faint)	Yellow-brown (faint)
Tyrosine.........................	Colorless	Colorless
Catechol.........................	Yellow	Red
Epinephrine......................	"	"
3,4-Dihydroxyphenylalanine........	"	"
Resorcinol.......................	Yellow-brown	Dark brown
Orcinol..........................	Yellow (faint)	Yellow-brown (faint)
Pyrogallol.......................	Dark brown	Dark red-brown
Phloroglucinol...................	Orange ppt.	Yellow-brown solution

Use of Wratten Filter—The color produced by 3,4-dihydroxyphenylalanine is an orange-red; that produced by catechol is a bright red. These colors can be matched by using the green Wratten No. 61 filter. The absorption spectra for the colored compounds and the transmission curve for the filter are shown in Fig. 1. The extinction coefficient is defined by the equation

$$\text{Extinction coefficient} = \frac{1}{cd} \log \frac{1}{n}$$

where c is the concentration in moles per liter (assuming 1 molecule of colored compound per molecule of catechol or 3,4-dihy-

droxyphenylalanine), d is the length in cm. of the column of fluid through which the light is passing, and n is the fraction of the incident light transmitted by d cm. of colored fluid. It will be observed that the wave-length of maximum light absorption of the colored solutions (510 $m\mu$) is close to the wave-length of maximum light transmission of the filter (530 $m\mu$). The absorption data were obtained with a Bausch and Lomb spectrophotometer.

Specificity of the Reaction—Table II indicates that compounds containing only one phenolic OH group react weakly or not at all with the reagents. Compounds having two or three phenolic OH groups react strongly, orcinol being an exception to this rule.

Stability of the Color—The color is stable for at least 1 hour, but fades if left overnight.

Determination of Tyrosine

Reagents—

1. Mercuric sulfate reagent. Dissolve 15 gm. of mercuric sulfate in 100 cc. of 5 N sulfuric acid.

2. Nitrite reagent. Dissolve 0.2 gm. of sodium nitrite in 100 cc. of distilled water.

3. Standard solution. Dissolve 100 mg. of pure tyrosine in enough distilled water to make a volume of 1 liter. Preserve under toluene.

Procedure—Place 1 cc. of unknown tyrosine solution (containing 0.05 to 0.15 mg. of tyrosine) in a test-tube graduated at 5 cc. Place 1 cc. of standard solution in a similar test-tube. Add to each tube 1 cc. of mercuric sulfate reagent. After mixing well, immerse both tubes in a boiling water bath for 10 minutes. Cool and add 1 cc. of nitrite reagent to each tube. Add enough distilled water to make a volume of 5 cc. If 3,4-dihydroxyphenylalanine is present, the solution will be turbid. Centrifuge until clear; pipette off 3 to 4 cc. of the clear, red supernatant liquid, and compare with the standard in a Duboscq colorimeter. If centrifugation is not done, wait 5 to 10 minutes after the addition of the nitrite reagent before comparing in the colorimeter. The author has less difficulty in matching greens than reds and has used the Wratten filter in obtaining his readings. The absorption spectrum of the tyrosine derivative is given in Fig. 1.

Results obtained by analyzing both pure tyrosine solutions and tyrosine-3,4-dihydroxyphenylalanine mixtures are recorded in Tables III and IV.

Discussion—The determination of tyrosine is based on the Millon reaction. The procedure described is a modification of the methods reported by Folin and Ciocalteu (2) and by Folin and Marenzi (5). The phenol reagent (2–4, 7) cannot be used in the analysis of mixtures, since both tyrosine and 3,4-dihydroxyphenylalanine give the reaction.

TABLE III

Determination of Tyrosine in Pure Solutions

Theoretical concentration	Determined concentration	Theoretical concentration	Determined concentration
mg. per l.	*mg. per l.*	*mg. per l.*	*mg. per l.*
50.0	51.8	110.0	109.9
70.0	70.9	130.0	129.8
90.0	90.1	150.0	147.1
100.0	100.9		

TABLE IV

Determination of Mixtures

Solution No.	Tyrosine		3,4-Dihydroxyphenylalanine	
	Theoretical concentration	Determined concentration	Theoretical concentration	Determined concentration
	mg. per l.	*mg. per l.*	*mg. per l.*	*mg. per l.*
1	60.0	61.0	105.0	104.0
2	100.0	100.0	75.0	75.0
3	140.0	142.0	45.0	44.0
4	60.0	60.1	30.0	29.5
5	80.0	79.3	75.0	75.5

Effect of 3,4-Dihydroxyphenylalanine on Tyrosine Analysis— If 3,4-dihydroxyphenylalanine solution and the mercuric sulfate reagent are mixed and immersed for 10 minutes in a boiling water bath, the solution assumes a faintly yellow color. After cooling, this yellow compound precipitates, forming a cloudy solution. The addition of the nitrite has no effect on this precipitate; centrifugation is sufficient to remove it quantitatively.

Changes in Intensity of Color with Time—For about 4 or 5

minutes after the addition of the nitrite reagent the color steadily increases. After this it remains constant for at least 1 hour, but fades overnight.

SUMMARY

1. A colorimetric method for the quantitative determination of 3,4-dihydroxyphenylalanine in the presence of tyrosine is described. The method is based on the observation that an acid solution of 3,4-dihydroxyphenylalanine reacts with a nitrite-molybdate reagent to give a compound having a yellow color, this color changing to red on addition of excess alkali.

2. A colorimetric method for the quantitative estimation of tyrosine in the presence of 3,4-dihydroxyphenylalanine is described. The method is a modification of the Millon reaction.

3. Absorption spectra of the various colored solutions and the transmission curve of the green Wratten No. 61 filter are included.

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THE DETERMINATION OF SERUM CALCIUM BY TITRATION WITH CERIC SULFATE

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In the most commonly employed titrimetric methods for determination of calcium in blood serum a very dilute standard potassium permanganate solution is used, which possesses the disadvantages of instability, sensitivity to light, and the necessity for tedious labor and utmost care in preparation when especially stable solutions are required (1). In recent years it has become well known that ceric sulfate readily overcomes these difficulties and is of further advantage over potassium permanganate in that the valence change is simple, $Ce^{++++} + e \rightarrow Ce^{+++}$, the only possible change, and in that hydrochloric acid does not interfere (2). Moreover, the development of o-phenanthroline ferrous complex as an internal, reversible, high potential oxidation-reduction indicator has made its use widely and easily applicable (3).

Ceric sulfate has been used in the microdetermination of blood calcium by Rappaport (4). He uses it in excess to liberate iodine which is titrated back with standard 0.01 N sodium thiosulfate, thus requiring an additional standard solution. The writer has adapted the method of Willard and Young (*cf.* (5) p. 57) to the determination of 0.2 mg. of calcium as calcium oxalate, requiring but one standard solution. Calcium oxalate is dissolved in 4 M hydrochloric acid and iodine monochloride (6) is added so that the reaction will take place at a lower temperature. The oxalate is titrated with 0.01 N ceric sulfate at 45–50° in the presence of o-phenanthroline indicator. The color change is from pink to pale blue. The blue is so faint as to make the end-point pink to practically colorless. To keep the solution warm and to make the color change easily apparent, the titration is conducted in a white enameled water bath.

Standardization of 0.01 N Ceric Sulfate

Reagents—

1. 0.01 N sodium oxalate. 0.6700 gm. of Sørensen's or Bureau of Standards' sodium oxalate, dried for 1 hour at 120°, is dissolved and diluted to 1000 ml. It is prepared on the day it is to be used.

2. 0.1 N ceric sulfate ((5) p. 52).[1] (a) Weigh 66 to 67 gm. of anhydrous ceric sulfate into a 600 ml. beaker and add 28 ml. of concentrated H_2SO_4. Dilute with 28 ml. of water and stir, with frequent additions of water and heating, until all the salt is dissolved. Transfer to a 1000 ml. volumetric flask and dilute to the mark. Or (b) dissolve 80 gm. of ceric ammonium sulfate ((5) p. 52) in a 600 ml. beaker by the addition of 28 ml. of H_2SO_4 diluted to 500 ml. with water. Stir until all is dissolved. Transfer to a 1000 ml. flask and dilute to the mark.

3. 0.01 N ceric sulfate. In a 600 ml. beaker add 28 ml. of sulfuric acid to 400 ml. of water. Add 100 ml. of 0.1 N ceric sulfate. Transfer to a 1000 ml. volumetric flask and dilute to the mark.

4. Iodine monochloride catalyst (6).[1] Prepare the following solutions: (a) 0.005 M KIO_3 (1.07 gm. dissolved in 1000 ml. of water); (b) 0.01 M KI (1.66 gm. dissolved in 1000 ml. of water). Dissolve 10 gm. of potassium iodide and 6.74 gm. of potassium iodate in 90 ml. of water. The solution should not liberate iodine. Add 90 ml. of concentrated hydrochloric acid and stir well. The solution will be orange-colored. Transfer to a separatory funnel and add 5 ml. of chloroform. If the potassium iodide has been added in slight excess, the chloroform will be colored faintly red. Add a few drops at a time of the dilute potassium iodate until the chloroform is faintly pink. By various treatments with the balancing solutions the proper quantities of reagent are ascertained. The solution is 0.5 M in iodine monochloride. Store in a glass-stoppered bottle in the dark. Dilute 0.1 ml. to 10 ml. as required for use.

5. o-Phenanthroline indicator 0.025 M (3).[1] (a) o-Phenanthroline monohydrate, $C_{12}H_8N_2 \cdot H_2O$, 14.85 gm.; (b) ferrous sulfate 0.025 M, 6.95 gm. of $FeSO_4 \cdot 7H_2O$ in 1000 ml. of solution. Dis-

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[1] These prepared solutions are obtainable from The G. Frederick Smith Chemical Company, Columbus, Ohio.

solve the ferrous sulfate and add the *o*-phenanthroline and stir until all is dissolved, giving a dark red solution. Dilute 1:100 with water before use. The 0.025 M solutions will keep unchanged at least 1 year. The diluted indicator will last 3 to 4 days.

6. Hydrochloric acid, concentrated; sp. gr., 1.18 to 1.19.

Standardization of Ceric Sulfate

To 4.00 ml. of 0.01 N sodium oxalate in a test-tube add 1.0 ml. of concentrated hydrochloric acid and 0.2 ml. of dilute iodine monochloride. Place in a white enameled water bath with the temperature at 45–50° for 1 minute. Add 3 drops of the diluted

TABLE I

Standardization of 0.01 N Ceric Sulfate

Solution	Deviation
N	N
0.009238	0.000004
0.009195	0.000047
0.009212	0.000030
0.009281	0.000039
0.009281	0.000039
0.046207	0.000159
0.009242 (Mean)	0.000032 (A.D.)

Percentage deviation of a single observation from the mean, 0.3 per cent.

indicator. Titrate with 0.01 N ceric sulfate until the pink color of the indicator no longer reappears after 30 seconds. The results are shown on Table I.

Ceric sulfate so prepared and standardized and stored in ordinary clear, glass-stoppered bottles without precautions against light changed by 0.1 per cent in a 4 week interval, as compared with the most stable preparation of potassium permanganate prepared according to Halverson and Bergeim, which changes 0.1 per cent per week in amber bottles in the dark ((1), (7) p. 768).

Determination of Serum Calcium

Calcium oxalate representing 2 cc. of serum is dissolved in 0.9 ml. of 4 M hydrochloric acid, 0.1 ml. of catalyst is added, and

the solution titrated as given in the standardization procedure, with 1 drop of the diluted indicator.

Calculation—

$$\frac{20 f (A - B)}{V} \quad \text{mg. Ca per 100 ml. serum}$$

A = ml. ceric sulfate or potassium permanganate used in titration
B = "   "   "   "   "   "   "   " blank
V = volume of serum represented by sample
f = ratio of volume of standard 0.01 N sodium oxalate to volume of 0.01 N ceric sulfate or potassium permanganate used in the standardization

Blank determinations were performed in all standardizations and determinations. For both ceric sulfate and potassium permanganate the blanks were between 0.01 and 0.02 ml.

All glassware was scrupulously cleaned with dichromate-sulfuric acid cleaning mixture. Titrations were made with a 5 ml. burette graduated in 0.02 ml. divisions which could be estimated to 0.004 ml.

<div align="center">EXPERIMENTAL</div>

Comparison of Results with Those by Permanganate Titration

Calcium determinations on the same serum specimen were made by both ceric sulfate and potassium permanganate titrations. In these analyses the calcium was precipitated and washed as follows.

To 1 volume of serum were added 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid, slowly and with constant shaking. After standing for half an hour, the mixture was filtered through ashless filter paper. 10 ml. of filtrate, equivalent to 2 ml. of serum, were pipetted into a conical centrifuge tube. 1 drop of methyl red-methylene blue indicator was added and 20 per cent sodium hydroxide added dropwise until the solution turned pale green. In order to prevent precipitation of magnesium oxalate, 5 per cent acetic acid was added until the red color was restored. 1 ml. of 4 per cent ammonium oxalate was added and the contents of the tube were stirred with a footed stirring rod, the rod being washed down with distilled water. After standing overnight, the tube was centrifuged at high speed for 15 minutes (8). The supernatant fluid was drawn off

and the precipitate and sides of the tube were washed twice with 10 ml. portions of saturated calcium oxalate solution as suggested by Stanford and Wheatley (9), by the technique of Halverson and Bergeim ((1), (7) pp. 771, 772). The ceric sulfate titration was conducted as given above.

For the permanganate titration, the precipitate was dissolved in hot 1 N sulfuric acid and titrated with 0.01 N potassium permanganate standardized the same day against freshly prepared 0.01 N sodium oxalate. The results are given in Table II.

TABLE II
Calcium Determinations

By ceric sulfate	Deviation	By potassium permanganate	Deviation
mg. per cent	mg. per cent	mg. per cent	mg. per cent
12.87	0.03	12.84	0.02
12.94	0.04	12.76	0.06
12.97	0.07	12.73	0.09
12.89	0.01	12.88	0.06
12.80	0.10	12.90	0.08
12.93	0.03	12.74	0.08
		12.87	0.05
77.40	0.28		
12.90 (Mean)	0.05 (A.D.)	89.72	0.44
		12.82 (Mean)	0.06 (A.D.)

Deviation of single observation from mean, 0.4%	Deviation of single observation from mean, 0.5%

The difference between the two sets of results is 0.08 mg. per cent or 0.6 per cent.

SUMMARY

A method is described whereby 0.2 mg. of calcium as calcium oxalate may be determined by titration with standard ceric sulfate, with iodine monochloride as catalyst and o-phenanthroline as indicator. The percentage deviation of a single observation from the mean is 0.4 per cent.

The method is of advantage over the analogous potassium permanganate titration in that the reagents employed possess greater stability.

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STUDIES ON THE CATALASE AND PEROXIDASE ACTIVITY OF THE LIVER CELL

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Regenbogen (1), in developing his thesis of the antagonism between catalase and peroxidase, presented data to show that the catalase activity of washed liver and kidney tissue (in phosphate buffer solutions at 37°) was greatest at pH 5.50 and diminished with increasing alkalinity, becoming zero above pH 7.73. This is contrary to the results obtained by Morgulis, Beber, and Rabkin (2) with a partially purified catalase preparation from beef kidney. To determine the effect of hydrogen ion concentration on catalase under conditions approaching more closely those occurring in the tissue, we experimented with intact liver cells.

The livers of adult white rats freed from blood by perfusion with Tyrode's solution were sliced and pressed through several layers of gauze to separate the parenchymatous tissue from the ducts and connective tissue. By washing with Tyrode's solution and centrifuging uniform suspensions of liver cells were finally obtained.

To duplicate as nearly as possible the conditions of Regenbogen's investigation, the catalase activity of the liver cell suspension was measured in phosphate buffer solutions at 37°. Cell suspensions in a 0.003 M phosphate buffer mixture of the desired pH were kept 3 hours at 37° with occasional shaking. Sufficient H_2O_2 was then added to make the final concentration 0.29 N. The reaction was stopped after 30 minutes by adding N H_2SO_4. The unchanged H_2O_2 was determined iodometrically.

The relative peroxidase activity was determined on similarly treated cell suspensions which were allowed to react 30 minutes with pyrogallol and H_2O_2. The purpurogallin formed was ex-

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tracted with ether and determined colorimetrically, a correction being made for the color developing in a control sample. The results are recorded in Table I.

The sharp decline in catalase activity with increasing alkalinity observed by Regenbogen fails to appear under these conditions. Furthermore, Regenbogen found no purpurogallin below pH 7.4, whereas in our experiments the formation of purpurogallin extends to about pH 6.40.

Since catalase undergoes destruction at all temperatures above 2°, it is probable that at 37° the amount of H_2O_2 decomposed is determined not only by changes in activity of catalase at different pH values but also by the amount of catalase destroyed by the H_2O_2. To eliminate the latter factor we carried out determinations at 2°. Instead of the phosphate buffer Tyrode's solution

TABLE I

Catalase and Peroxidase Activity of Liver Cells

Total H_2O_2 used, 248.5 mg.; total pyrogallol, 250.0 mg.

pH....................................	5.50	6.09	6.40	7.00	7.49	7.92
H_2O_2 decomposed, *mg*............	47	60.9	58.2	43.7	38.9	28.9
Per cent of total................	18.9	24.5	23.4	18.0	15.6	11.6
Purpurogallin, *mg*...............	0	0	0.04	0.94	1.07	1.05

was used, adjusted to the desired pH by varying the CO_2 and $NaHCO_3$ content (3).

The liver cell suspension in Tyrode's solution was kept, as before, 3 hours at 2°, when H_2O_2 was added to 0.02 N concentration, and the reaction allowed to proceed 30 minutes with constant shaking. The pH of the liver cell-Tyrode mixture was determined with the quinhydrone electrode immediately before the addition of the H_2O_2.

The results under these conditions show that within a pH range of 6.38 to 7.80 catalase activity, unlike that in the phosphate buffer, varies insignificantly (84 to 89 per cent of the H_2O_2 decomposed), and is in good agreement with our previous findings (2). We found no evidence of a sharp decline in catalase activity between pH 7.16 and pH 7.38, as claimed by Regenbogen.

SUMMARY

Our experiments do not seem to support Regenbogen's contention of the reciprocal relationship between the catalase and the peroxidase activity which corresponds to an anerobic-aerobic rhythm of the life of the cell.

The catalase activity of the rat liver cell in Tyrode's solution at 2° shows practically no variation within the range of pH 6.38 to 7.82.

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CHEMICAL COMPOSITION OF THE BLOOD OF THE HEN DURING ITS LIFE CYCLE

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Previous publications from this laboratory have reported that the total phosphorus as well as certain organic and inorganic fractions found in cells and serum changed with the age of the hen, especially at the time of egg production and at the molting season (1). Similar studies of the calcium distribution demonstrated that there were also changes in certain fractions of the calcium at corresponding periods in the life cycle of the hen. This phenomenon, which has not been observed in mammals, suggested that there might be changes in other chemical constituents of the blood of the fowl. An investigation of the literature failed to reveal any complete studies which might be used for comparisons. Many isolated reports gave certain components without reporting methods, age or breed of hen, or the ration being consumed, or whether or not the hen was laying. Karr and Lewis (2) referred to the urea distribution. Scheunert and Pelchrzim (3) compared the sugar, nitrogen, creatinine, and uric acid of the hen with other animals. Pupilli (4) gave the average uric acid and urea content of the blood of the hen. Hayden and Fish (5) gave the average finding for certain constituents at given ages of a definitely named breed of fowl, while the most complete report by Dyer and Roe (6), which appeared during the progress of this investigation, gave more complete data but no information in regard to life cycle changes. Table I gives the findings for a known variety of chickens at a definite age and eating a definite ration throughout their cycle. Any changes that might have occurred at the time of egg production and period of molting were carefully observed.

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TABLE I

Analysis of Chicken Blood

L., laying; N., non-laying.

	Age	Urea N	Non-protein N	Creatinine	Uric acid	Glucose	Sodium	Plasma chloride	Cell chloride	Total solids	Ash	Refractive index
						Mg. per 100 cc.						
	mos. 1934											
	1. May	3.05	45.8	1.29	4.80	231	337	393	175	14.4	1.06	1.3426
	2. June	3.14	35.8	1.11	4.18	244	354	406	173	14.0	1.13	1.3437
	3. July	2.62	49.5	1.36	4 72	235	348	395	199	14.2	1.06	1.3435
	4. Aug.	2 93	46.1	1.20	4.25	252	364	418	210	14.9	1.15	1.3434
	5. Sept.	3.06	47.2	1.34	4.67	227	352	408	188	14.9	0.77	1.3440
	6. Oct.	4.28	48.5	1.20	4.13	211	355	417	170	14.0	0.91	1.3452
	7. Nov.	4.39	42.5	0.90	4.04	201	340	392	152	14.6	0.92	1.3455
L.	8. Dec.	4.53	38.5	1.44	3.25	212	341	390	150	14.2	0.90	1.3450
N.		4.90	45.5	1.47	4.81	227	344	385	174	14.2	0.98	1.3471
	1935											
L.	10. Feb.	4 08	44.5	1.50	3.63	232	340	407	160	16.1	0.87	1.3480
N.		3.88	49.3	1.49	3.88	233	349	352	177	20.7	0.92	1.3456
L.	11. Mar.	4.20	44.6	0.98	7.61	204	352					1.3495
N.		4.10	36.6	1.27	6.88	212	333					1.3520
L.	12. Apr.	2.40	41.9	1.05	5.10	196	337	401	165	18.5	0.95	1.3506
N.		2.50	33.5	0.92	6.01	189	342	391	172	16.5	0.92	1.3498
L.	13. May	4.10	43.5	1.10	4.76	185	339	380	161	19.7		1.3463
N.		3.30	42.0	0.90	5.48	176	342	376	201	19.8		1.3521
L.	14. June		42.9	1.00		191				19.2	1.02	1.3517
N.			43.1	0.90		192				17.1	0.97	1.3539
L.	15. July	2.90	52.9	1.29	3.67	186				18.5	0.84	1.3537
N.		2.80	57.1	0.99	5.80	184				18.1	0.85	1.3479
L.	17. Sept.	3.10	43.7	1.29	6.56	191	335	392	164	15.7	0.87	1.3458
N.		2.20	60.1	1.48	5.64	171	341	387	182	17.0	0.82	1.3418
L.	18. Oct.	2.83	48.8	1.52	6.30	205						
N.		2.62	55.5	1.48	5.96	184						
L.	19. Nov.	2.20	42.3	1.59	5.47	199	332	395	162	16.5	0.89	1.3502
N.		2.56	45.7	1.65	4.88	183	337	382	187	17.6	0.87	1.3452
L.	20. Dec.	2.62	57.1	1.73	4.27	217						
N.		3.56	62.1	1.53	4.36	190						
	1936											
L.	21. Jan.	4.94	64.0	1.08	5.16	202						
N.		5.62	61.0	1.06	5.56	213						

TABLE I—*Concluded*

	Age	Mg. per 100 cc.										Refrac- tive index
		Urea N	Non-protein N	Creatinine	Uric acid	Glucose	Sodium	Plasma chloride	Cell chloride	Total solids	Ash	
	mos. 1936											
L.	22. Feb.	3.64		1.67	5.44	197						
N.		4.97		1.50	3.73	202						
L.	23. Mar.	3.46	58.3	1.18	5.51	192						
N.		3.82	60.6	1.13	4.46	219						
L.	24. Apr.	4.02	33.6	0.93	4.04	196						
N.		4.80	40.8	0.94	5.13	208						
L.	25. May	3.22	28.0	0.92	4.51	181	350	408	165	17.7	0.81	1.3482
N.		3.73	59.2	0.91	6.06	199	346	392	172	16.4	0.73	1.3494
L.	26. June	3.92	46.3	1.09	5.30	180						
N.		4.22	63.0	1.08	4.37	170						
L.	27. July	4.17	34.8	1.02	3.94	191	350	380	170	15.6	0.89	1.3479
N.		4.66	40.6	1.15	5.00	177	342	376	182	16.1	0.93	1.3418

EXPERIMENTAL

The chickens used were Rhode Island Reds, hatched in an incubator, reared for 1 month in a brooder, and transferred to a graveled yard. From this lot, 50 baby pullets were selected and fed a mash composed of 200 parts of bran, 100 parts each of yellow corn-meal, oats, and shorts, 75 parts of meat scraps, 50 parts of alfalfa leaf meal, 40 parts of cottonseed meal, 15 parts of dried buttermilk, 12 parts of bone meal, and 6 parts of salt. A little green feed was added daily. These data were needed for purposes of comparisons. Therefore, it was thought best not to fast the chickens previous to taking the blood samples, but to set up standards under normal conditions. At approximately monthly intervals, during a 2 year period, blood was drawn by heart puncture, early in the morning, from at least five of the chickens. The samples were delivered in iced containers, pooled, and analyzed. The data in Table I represent the average of from two to five analyses of such samples. The methods of determining the phosphorus and calcium distributions in the cell plasma or serum and the results obtained are omitted in this article, as they

were found to be similar to the data previously reported (1). The non-protein nitrogen and creatinine were determined by the method of Folin and Wu (7); urea according to Folin and Svedberg (8); uric acid, Folin (9); glucose, Folin and Wu (10); chlorides, Whitehorn (11); sodium, Butler and Tuthill (12); and the solids, ash, and refractive indices by regularly accepted methods.

DISCUSSION

An analysis of the data presented in Table I demonstrates that there are no striking changes in any of these blood constituents at any time, such as was reported to be true in the previous studies for both the organic and inorganic fraction of the calcium and phosphorus of the cells and plasma.

There are minor changes in some compounds as the chicken matures that may represent significant changes in the regularity of their occurrence. Urea nitrogen and the non-protein nitrogen are practically the same irrespective of age. The urea nitrogen is less, and the total nitrogen and uric acid more than were reported by Hayden and Fish (5), and compare more favorably with those of Dyer and Roe (6). The urea nitrogen is always lower than the usual figure reported for domestic animals. The creatinine is quite constant and compares favorably with that of other animals. Uric acid seems to be independent of age, the variation probably depending upon the food intake. The glucose decreased somewhat with the age of the hen. No regular difference between laying and non-laying was found. The amount was always far in excess of that reported by Hayden and Fish (5), and slightly above that presented by Burrows, Fritz, and Titus (13).

The determinations for the total solids, ash, sodium, chlorides in cells and plasma, and the refractive index of the serum, show only small daily variations, as have been noted for other animals in this laboratory. Obviously, the only striking changes are to be found in the calcium and phosphorus fractions and these factors have undoubtedly been developed in the course of the evolution of the hen to provide for the unusual burden placed upon it at the time of egg production.

SUMMARY

1. No significant changes were noted in any of the constituents determined during the first 2 years of the hen's life.

2. Urea is lower, uric acid and glucose higher, while other constituents resemble the percentages found in the blood of other domestic animals.

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I

SUBSTANCES WHICH INHIBIT COLOR DEVELOPMENT IN THE SULLIVAN METHOD FOR CYSTINE*

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The very useful method devised by Sullivan (1–4) for the determination of free cystine has been widely used for some years and ranks as the most specific method known for the purpose. For this reason whenever the results obtained by it on biological materials have been lower than those of other procedures, the conclusion has usually been drawn that the sample under investigation contained other disulfide compounds besides free cystine and that, whereas such methods as. that developed by Folin and Marenzi (5) gave the sum of all disulfide and sulfhydryl compounds, the Sullivan method could be relied on to give an accurate measure of the free amino acid only. However, during the past few years various workers have criticized the accuracy of the results obtained by the original Sullivan technique and have suggested various modifications. Among such studies may be cited those of Lugg (6) and Rossouw and Wilken-Jorden (7). Zahnd and Clarke (8) have observed failure on the part of the cystine extracted by butyl alcohol to give the Sullivan reaction. This failure was later explained as being due to the presence of aldehydes (9). Meldrum and Dixon (10) have reported inhibition of the Sullivan reaction in the presence of reduced glutathione. Their results were confirmed by Sullivan and Hess (11) who recommended certain changes in the reagents used (an increase in the amount of both cyanide and naphthoquinone) as a means of overcoming this difficulty. In a recent paper Lee (12) criti-

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* A preliminary report of this work was presented at the Thirtieth meeting of the American Society of Biological Chemists at Washington, March 25–28, 1936 (*Proc. Am. Soc. Biol. Chem.*, **8**, iv (1936); *J. Biol. Chem.*, **114** (1936)).

cized the results obtained by the original Sullivan method on casein hydrolysates and reported more satisfactory results by the Lugg modification, in which the final solution is more highly buffered. Furthermore, a cystinuric dog urine has been reported by Green *et al.* (13) in which no Sullivan color could be obtained until the cystine had been separated by the cuprous mercaptide method (14). The effect of homocystine in depressing color formation in the Sullivan method (but not in the Lugg modification) has been reported by both Brand *et al.* (15) and by Lewis, Brown, and White (16). It should be noted, however, that Sullivan and Hess (4) fail to confirm this finding. Thus there has been considerable accumulation of evidence that certain substances can interfere with the development of color in this method. Such interference was mentioned by Sullivan (1) on the part of such compounds as pyrogallol, hydroquinone, etc., because the colors produced by these substances in the method are not discharged by sodium hydrosulfite. He also stated in this paper that hydrogen sulfide interferes with color development and advanced as an explanation a reduction of the dye, in spite of the large excess in which the latter is used. The criterion of interference used by Sullivan with regard to most substances (except H_2S) appears to have been the formation of an extraneous color which was not discharged by hydrosulfite and which therefore might produce high results. In a later paper (3) he stated "The presence of reducing material, such as H_2S, is to be avoided, since it interferes with the progress of the color reaction." However, the type of interference by which such a compound would greatly *reduce* the color given by a certain amount of cystine seems to have received little consideration. Brand, Harris, and Biloon (17) in a paper on cystinuria pointed out that the apparent cystine content of freshly passed cystinuric urines, as determined by the Sullivan method, rose as the sample stood exposed to the air and gradually reached a moderately constant value. This phenomenon was attributed by Brand and coworkers to the excretion of a complex (possibly a peptide) of cystine to which the Sullivan method would not respond and which quickly hydrolyzed to produce an additional amount of free cystine. Similar observations were reported by Hickmans and Smallwood (18). Andrews and Randall (19), while confirming this observation on

cystinuric urines, suggested that some influence on the Sullivan reaction might be responsible for this drift in the cystine value. A similar suspicion was voiced by Brand, Cahill, and Block (15) who suggested as one possibility that ascorbic acid (vitamin C) in the urine might interfere with color development in this method and that its oxidation, as the sample stood exposed to air, would result in gradual removal of the inhibitory effect and produce higher cystine values. A recent communication by Brand *et al.* (20) reports that both thioglycolic and dithioglycolic acids markedly depress color formation in both the Sullivan and Lugg-Sullivan reactions.

The procedure used by Brand and coworkers in running Sullivan determinations on urine (17) is practically that originally recommended by Sullivan. Similar conditions were employed by Csonka (21) for the determination of cystine in glutelin hydrolysates. The technique outlined by Brand and coworkers has been very generally used by other workers as being the most concise and convenient set of directions available. The exact timing of the reaction has received considerable attention. This is particularly true of the stage between addition of the naphthoquinone and of the sodium sulfite solution. Sullivan (3) recommended 10 seconds shaking between these two reagents, while in the method as described by Brand, Harris, and Biloon it is merely specified that the sodium sulfite solution should be added "immediately" and "as quickly as possible." The studies of Rossouw and Wilken-Jorden (7) emphasize the fact that, if "immediate" addition means within less than 5 or 10 seconds, highly variable amounts of color can result. When (on a 25 ml. basis) 2 ml. of 5 per cent sodium cyanide are used, it would appear from Rossouw and Wilken-Jorden's curves that a 20 second interval at this point would give a maximum color and be a safer procedure. We have accordingly used an interval of 20 seconds in our work. Otherwise, our procedure has been that described by Brand, Harris, and Biloon (17).

A few preliminary experiments in which varying amounts of ascorbic acid were added to samples of cystinuric urine showed that comparatively small amounts (a few mg. per cent) completely inhibited color formation by the Sullivan method (Brand modification). Furthermore, by aerating the sample at room tempera-

ture for 1 or 2 days the ascorbic acid was oxidized to such an extent that the sample again gave a Sullivan color practically equal to that originally produced. To apply the test in the opposite way, we added free cystine to a freshly voided sample of normal urine and compared the resulting Sullivan values for its cystine content when determined at once and after aerating. The determination run at once gave values of only about 22 per cent of the amount of cystine added. 24 hours aeration raised this to 76 per cent and 24 hours more aeration raised it to 80 per cent. It is clear therefore that we are not dealing with any cystine complex, since in the latter case free cystine itself was added. Moreover, the amounts of ascorbic acid normally present in fresh urines are ample for interference with the development of color in the Sullivan method as it has been used in the past. Similar effects with ascorbic acid have been reported by Brand, Cahill, and Block (15).

These results impelled us to examine a variety of other reducing substances for their ability to interfere with the method. The procedure employed consisted in placing constant amounts of cystine in a series of flasks and adding the same reagents to each plus either the solution of the substance to be tested or, for the standard, an equal volume of water. The standard was set at 20.0 mm. in the colorimeter and the other solutions matched against it. It was found convenient to make up the solutions in volumetric flasks to 100 ml. instead of the 25 ml. used by Brand and others. The procedure used was therefore as follows: 2.0 ml. (8.0 mg.) of cystine in 0.1 N HCl; 25.0 ml. of inhibitor solution (or water); 10.0 ml. of 5 per cent aqueous sodium cyanide, 10 minutes standing; 4.0 ml. of 0.5 per cent sodium β-naphthoquinone-4-sulfonate, 20 seconds standing; 40.0 ml. of 5 per cent anhydrous sodium sulfite in 0.5 N sodium hydroxide, 30 minutes standing (in the dark); 4.0 ml. of 2 per cent sodium hydrosulfite in 0.5 per cent sodium hydroxide; 0.5 N sodium hydroxide to volume (100 ml.).

The solutions were read at once after being mixed. In reading these solutions we have found it advantageous to employ in the eyepiece of the colorimeter a Wratten filter (Eastman Kodak Company) No. 62 with maximum transmission at wave-length 530 $m\mu$. By its use the accuracy of readings in Sullivan determi-

nations of cystine is much enhanced. Nearly 100 substances were examined in this way for their ability to inhibit the development of color. These included a wide variety of organic substances, but it soon became evident that only those having reducing properties were active inhibitors. The most active were adrenalin, ascorbic acid, the various photographic developers, tannic acid, hydrogen sulfide, and certain cyclic cystine derivatives, the most active of which was cystine hydantoin. The
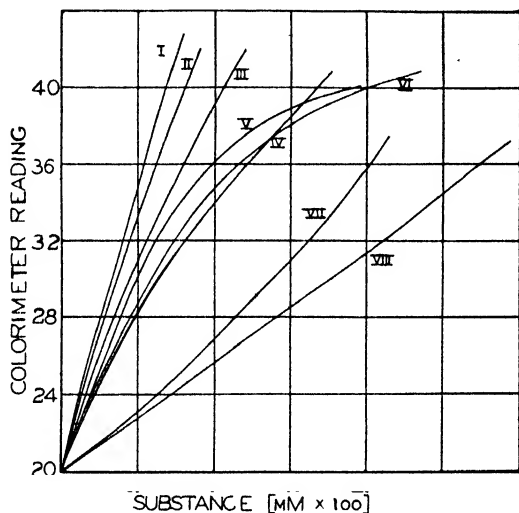
Fig. 1. Inhibitive effect on color development in the Sullivan method for cystine of cystine hydantoin, Curve I; adrenalin, Curve II; hydroquinone, Curve III; tannic acid, Curve IV; pyrogallol, Curve V; diamidophenol, Curve VI; hydrogen sulfide, Curve VII; ascorbic acid, Curve VIII.

ability of some of these compounds to change the colorimeter reading obtained from constant amounts of cystine by the method described above is shown in Fig. 1. In this figure the amount of inhibitor in each 100 ml. determination containing 8.0 mg. of cystine is plotted in hundredths of a mm. It is obvious that comparatively small amounts of these inhibitors suffice to produce very erroneous figures for the cystine content of the solution. In conducting these tests, various samples of the same inhibitor from different sources were taken. For example, in the case of

adrenalin three different preparations (one of the hydrochloride, one of the bitartrate, and one of the free base) were used. In the tests on cystine hydantoin two samples made by the cyanate method and one prepared from urea all gave identical results.

A number of other substances were found, chiefly among the milder reducing agents, which when present in larger quantities materially affect the method. These include the simple aldehydes, sugars, and sugar derivatives such as glycuronic acid and some of their conjugation products.[1] Fig. 2 shows the curves of a number of these substances again plotted in millimolar terms except that the abscissæ are expressed in mM instead of hun-
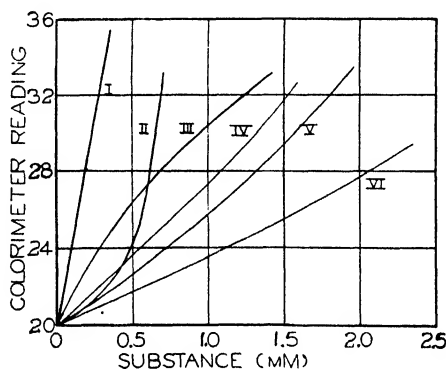
Fig. 2. Inhibitive effect on color development in the Sullivan method for cystine of fructose, Curve I; maltose, Curve II; galactose, Curve III; glucose, Curve IV; arabinose, Curve V; formaldehyde, Curve VI.

dredths of a mM. The amounts plotted in Fig. 2 are therefore 100 times greater than those in Fig. 1.

The curve produced by hydrogen sulfide (Fig. 1) is significant. The Sullivan method has often been applied to sulfur compounds so unstable that under the alkaline conditions of the method sulfides are easily split off. Cystine hydantoin is such a substance and its great effectiveness as an inhibitor is no doubt due to the ease with which it loses hydrogen sulfide as well as to the fact that the resulting hydantoin ring also has an inhibitive effect. It is evident that, with either hydrogen sulfide or any

[1] Kindly furnished by Dr. Armand J. Quick.

potential producer of the same present, a reliable measure of the free cystine content is not to be expected. The effect of sulfides, although mentioned in Sullivan's early papers (see above), appears to have been lost sight of (22).

A number of samples of freshly excreted normal urine from different subjects have been examined for inhibitive properties in the Sullivan reaction. We have, in all cases, found a degree of inhibition far larger than that accounted for by the ascorbic acid content alone, as indicated by indophenol titration. It appears that freshly excreted urine contains some substance or substances besides ascorbic acid of strong reducing properties. The unknown substance can be oxidized by aeration and can be completely removed by decolorizing carbon. The amounts of such reducing substances as the sugars in normal urines are far too small to account for these effects.

The recognition of this ability of reducing agents to produce low cystine values by the Sullivan method is of particular significance when compared with the results given by the Folin phosphotungstic method on similar materials. Sullivan and Hess (23) discuss at length the relative figures for the cystine content of different proteins as determined by the Sullivan and Folin colorimetric methods. The same question arises later (24) in connection with measurements of the rate of absorption of cystine from the gastrointestinal tract of the white rat. In this latter case determinations of unabsorbed cystine in the intestine of the rat gave different results by the two methods and, therefore, produced quite widely differing absorption rates. Determinations made by the Okuda bromate titration gave results intermediate between those of the Sullivan and the Folin methods but considerably closer to the Sullivan figures. In both of these discussions there is ample realization of the fact that other reducing substances than cysteine (even non-sulfur reducers) are capable of reducing the Folin reagent and that results by this method are very likely to be higher than those justified by the cystine content alone. However, although the rôle of reducing agents in raising the results of the Folin determination has been fully recognized, similar recognition of this rôle in lowering the results of the Sullivan determination seems to have been almost entirely lacking. It appears therefore that in such materials

the true cystine content is represented by neither the results of the one nor the other but lies somewhere between and that the higher the content of extraneous reducing materials the greater will be this magnified divergence between the results of the two methods. As an isolated example of inhibitory effect on the Sullivan method by a partial protein hydrolysate the following may be mentioned: A proteose-peptone mixture, resulting from partial hydrolysis of human hair by sulfuric acid, was tested as described above. 20 mg. of this mixture changed the colorimeter reading from 20.0 to 23.5 and 50 mg. changed the reading to 27.5. This solution contained no free amino acids. Addition of larger amounts of alkali than those used in the method as described above did not change the results. It is evident that such an effect cannot be attributed to the buffering capacity of the solution.

The above criticisms apply to the results yielded by the method as it has been used by most investigators for some years. It is obvious that any modifications which would remove such interference would greatly increase the usefulness of the method. The modified procedure recommended by Lugg (6) has been tested in the presence of some of the most active inhibitors listed above with the result that less interference has been encountered than in the original Sullivan procedure. The slope of the inhibition curves obtained by the Lugg modification is about one-half that shown in Fig. 1 for some of the substances tested. However, the greater complexity of the Lugg method as well as the fact that the color produced is much lighter than that from the same amount of cystine in the original Sullivan method makes its employment somewhat questionable. It should be noted that Lugg reports interference with color development in the presence of large amounts of certain other amino acids and ascribes this interference to their buffering capacity. Although we have confirmed his observations in this regard, we find that these effects are much slighter than those we have encountered with ascorbic acid and other substances shown in Fig. 1. It is also evident, from the variety of structures of substances which we have found to interfere, that buffer capacity cannot be the explanation of our results. The only property which our various inhibitors have in common is their reducing property.

Our results with ascorbic acid, obtained as described above, have been confirmed by Dr. M. X. Sullivan (private communication) who has kindly made some suggestions for overcoming these inhibitory effects. One such suggestion was that of using increased amounts of the naphthoquinone, since there might conceivably be some competition for the naphthoquinone between the cystine and the inhibitor. In view of the molar ratios involved this seems hardly likely. In a flask containing 8.0 mg. of cystine, 1.0 mg. of ascorbic acid, and 4 ml. of 0.5 per cent naphthoquinone the molar ratios (in the above order) are 5.8:1:13.5. This excess of the naphthoquinone would seem adequate but we have nevertheless run a series of tests in which the amount of naphthoquinone was varied while all other constituents remained the same as in the standard procedure outlined above. The results indicate that an increase in the amount of naphthoquinone (up to about 6 ml. of the 0.5 per cent solution) produces not an *increase* but a *decrease* in the resulting color. This anomalous fact has already been reported by Lugg (6). It would seem that no advantage results from increase in the proportion of naphthoquinone. The same result has been obtained in the presence of constant amounts of ascorbic acid.

As a further means of preventing such interference Sullivan and Hess (4) have suggested the use of 5 per cent sodium cyanide dissolved in 1.0 N sodium hydroxide instead of the aqueous 5 per cent sodium cyanide usually used. We find that the addition of the alkaline cyanide to the list of changed conditions produces very much more satisfactory results. The effect of the alkaline cyanide on the slope of the inhibition curves is shown in Figs. 3 to 5 for adrenalin, ascorbic acid, and tannic acid respectively. It is evident that, while color inhibition is still not absent, its effect is decidedly minimized. This is especially true of ascorbic acid, because of the flattening of the curve produced.

We have also investigated the effect of using the sodium cyanide in 2.0 N sodium hydroxide, curves for which are included in Figs. 3 to 5. The resulting curve is again flatter, although the improvement over the results obtained with 1.0 N sodium hydroxide is not great. In fact, with adrenalin the curves obtained with cyanide in 1.0 N and 2.0 N sodium hydroxide were practically coincident. We have also used conditions in which
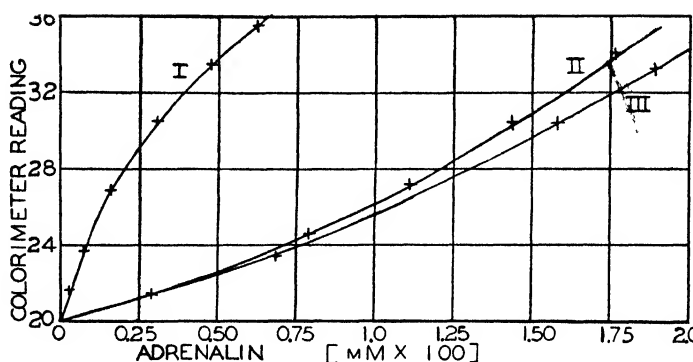
FIG. 3. Inhibitive effect of adrenalin on color development in the
Sullivan method for cystine.   The reduction of cystine effected by aqueous
sodium cyanide, Curve I; sodium cyanide in 1.0 N sodium hydroxide, Curve
II; sodium cyanide in 2.0 N sodium hydroxide, Curve III.
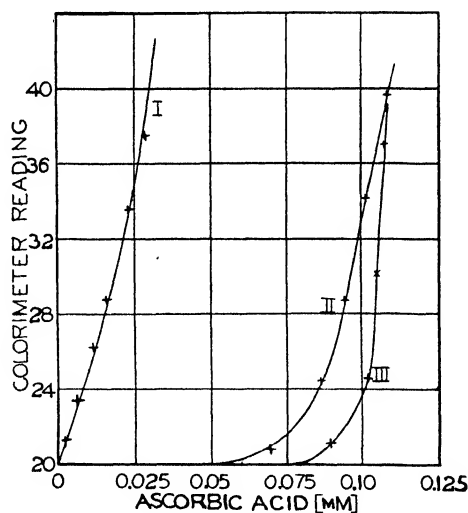
FIG. 4.   Inhibitive effect of ascorbic acid on color development in the
Sullivan method for cystine.   The reduction of cystine effected by aqueous
sodium cyanide, Curve I; sodium cyanide in 1.0 N sodium hydroxide, Curve
II; sodium cyanide in 2.0 N sodium hydroxide, Curve III.

not only the sodium cyanide but all subsequently used reagents were dissolved in 2.0 N sodium hydroxide but have found no improvement in the slope of these curves.

A series of experiments was made in which we have measured the effect of increased amounts of naphthoquinone solution when the recently modified conditions of Sullivan and Hess (4) were used. The results indicate that the decrease in color observed with increased amounts of naphthoquinone, when the modification of Brand, Harris, and Biloon (17) is used (see above), is no longer observed.
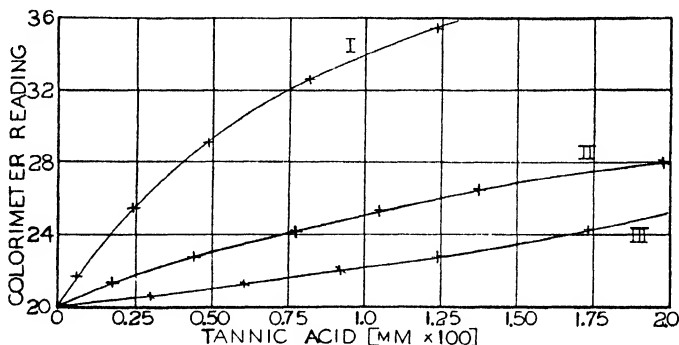
FIG. 5. Inhibitive effect of tannic acid on color development in the Sullivan method for cystine. The reduction of cystine effected by aqueous sodium cyanide, Curve I; sodium cyanide in 1.0 N sodium hydroxide, Curve II; sodium cyanide in 2.0 N sodium hydroxide, Curve III.

The experiments described above indicate that a variety of reducing substances produce low results, if the original Sullivan procedure is used. Since the method in this form has been widely used for some years, it seems appropriate to regard with suspicion many such figures now on record, especially those results, obtained by the Sullivan and the Folin methods, which show wide discrepancies. The modification recently proposed by Sullivan and Hess (4) has the merit of minimizing this interference to the point where the ordinary content of ascorbic acid in urine does not interfere with the cystine determination. However, we have found that even under these conditions freshly passed urine samples, in contrast to 24 hour samples, still cause a

marked diminution in the color obtained from constant amounts of cystine. Addition of 25 ml. of freshly passed urine to the standard 100 ml. determination described above (containing 8.0 mg. of cystine) has consistently produced readings indicating only from 60 to 70 per cent of the cystine actually present. This same result has been obtained from a number of different subjects. This may be taken to indicate the presence of some urinary constituent of such a reducing potential that even the modified conditions are unable to cope with it. It must therefore be emphasized that special care should always be taken in the employment of the Sullivan method that no such inhibitors are present. Oxidation of such bodies by aeration before the cystine determination is recommended, and any increase in the apparent cystine content over that obtained without aeration should be regarded as cause for suspicion. In the presence of objectionable amounts of inhibitors which are not susceptible to atmospheric oxidation, the procedure by which the cystine is separated as the cuprous mercaptide, followed by liberation and colorimetric estimation of the free cysteine (13, 14), is to be recommended.

SUMMARY

The Sullivan method for the determination of cystine in biological fluids produces low results in the presence of many reducing agents. Among these may be mentioned ascorbic acid, adrenalin, the photographic developers, hydrogen sulfide, and compounds producing sulfides under alkaline conditions. Aldehydes and simple sugars also inhibit color development in the Sullivan method, but larger amounts of these are required.

Failure to recognize this interference can result in erroneous figures for cystine content of various materials when such reducing substances are present in sufficient amount. This is particularly exemplified in the case of fresh urine samples which contain ascorbic acid and other substances which inhibit color formation in the Sullivan method.

A modification of the method, proposed by Sullivan, in which alkaline instead of aqueous sodium cyanide is used decidedly minimizes this interference but does not completely remove it.

Recommendations are made as to further means of avoiding such interference.

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THE LIPID AND MINERAL DISTRIBUTION OF THE SERUM AND ERYTHROCYTES IN THE HEMO- LYTIC AND HYPOCHROMIC ANEMIAS OF CHILDHOOD*

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The hemolytic anemias of early childhood, congenital hemolytic icterus, sickle cell, and erythroblastic, present not only clinical and hematological problems to the medical profession but also physicochemical ones to the biochemist and physiologist. The latter are especially concerned with the structure and function of the erythrocyte and cellular structure in general, particularly as they are related to growth and development, as pointed out in a preceding paper (1). These blood dyscrasias demonstrate anomalies in the production and destruction of erythrocytes as signified by abnormal red blood cells characteristic of each type of anemia (2). Defects in structure are indicated by the vulnerable spherical cell in hemolytic icterus (3, 4); the unique ability of the erythrocyte in sicklemia,[1] both with and without anemia, to assume the bizarre, thinned sickled forms under certain conditions (5, 6); the presence of erythroblasts and of mature cells with uneven distribution of hemoglobin in erythroblastic anemia (7–9); and the small

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* Presented before the American Society of Biological Chemists at its Twenty-ninth annual meeting at Detroit, April 10–13, 1935 (Erickson, B. N., Cope, F., Sternberger, H. R., Lee, P., Cooley, T. B., and Macy, I. G., *Proc. Am. Soc. Biol. Chem.*, **8**, xxx (1935); *J. Biol. Chem.*, **109** (1935)).

[1] Sicklemia is the term applied to the hematological anomaly in which the erythrocytes assume a sickle-like or crescentic shape *in vitro* under certain physical conditions. Sicklemia is manifested both without any accompanying clinical debilities and in sickle cell anemia which is characterized by distinct hematological and clinical symptoms.

TABLE I

Clinical and Hematological Characteristics of Hemolytic and Hypochromic Anemias of Childhood

	Erythroblastic anemia	Sickle cell anemia	Hemolytic icterus	Hypochromic anemia
Etiology	Mediterranean; hereditary and familial	Negro; hereditary and familial	Hereditary and familial	Nutritional
Type of erythrocyte	Large with uneven distribution of Hb; marked fragmentation thereby producing microcytes	Sickling in wet preparation; uneven distribution of Hb	Microcytic with tendency to spherocity; enlarged at crisis	Microcytic; deficient in Hb
Resistance to hemolysis	Increased to saponin and hypotonic NaCl	Increased to saponin and hypotonic NaCl	Decreased to saponin and hypotonic NaCl	May be slightly increased
White blood cell count	Increased; immature forms	Increased; immature forms	Increased; immature forms in severe crisis	Normal
Icterus index	Increased	Increased	Increased	"
Spleen	Always enlarged	Variable in size	Enlarged at crisis	"
Splenectomy	No improvement of anemia; remarkable increase of erythroblasts	No improvement of anemia; sickling unchanged	Anemia usually cured; increase of resistance but persistent microcytosis	

cell with deficient hemoglobin in hypochromic anemia (10).   The chief clinical characteristics of the different types of anemia concerned herein are summarized in Table I.

The hereditary anemias of childhood, characterized by extreme abnormalities in shape, size, weight, and fragility of the erythrocyte, offer opportunities for studying corpuscular structure and function by simultaneous hematological, physical, and chemical observations on the cells and plasma.   Inasmuch as the red blood cell may reflect the character of the parent tissue, such interrelated data during the course of the anemia may còntribute pertinent information on the inherent defects in the activity and development of the hematopoietic system.   Furthermore, such data may have clinical value.

The present study has been focused upon the concentration of the lipids, electrolytes, and protein in the erythrocytes and plasma, since the structure and function of the cell are dependent on these constituents and their synergism.

The analyses included sodium, potassium, chloride, nitrogen, and the complete lipid distribution in both cells and plasma.   The chemical studies were accompanied by comprehensive hematological and certain physical observations on the erythrocytes such as cell volume, weight, diameter, thickness, specific gravity, water content, and resistance to hemolysis against saponin and hypotonic sodium chloride solutions.   The lipid and mineral composition representative of an average single erythrocyte have been calculated as a more direct means of studying the relationship between its chemical and hematological properties.[2]

Methods

The hematological and physicochemical observations together with determinations of minerals and lipids in the serum and cells were carried out by the procedures described in the preceding paper on normal children (1).

TABLE II—*Hematological Observations on*
The subjects within each group of anemia are arranged according to the severity of the

Observation	Date	Subject	Sex	Age	Red blood cells		Hemoglobin		Hematocrit
					Whole blood	Per gm. cells	Whole blood	Single cells	
				yrs.	*millions per c.mm.*	$\times 10^{10}$	*gm. per 100 cc.*	*micro-micro-grams*	*per cent*
Normal children (average of 26 studies)					4.7	1.08	13	28	40
Sicklemia*									
Anemia	Apr. 29, 1936	C. D.	F.	5	2.2	0.95	5	26	20
	Mar. 18, 1935	"	"	4	2.5	0.82	7	28	28
	May 10, 1935	"	"	4	2.7	1.03	9	35	24
	Oct. 5, 1936	E. D.	M.	5	2.2	1.07	6	30	20
	June 16, 1936	"	"	5	2.5	1.00	6	26	23
	Feb. 18, 1935	A. B.	F.	14	2.0	0.92	7	36	20
	" 28, 1935	B. D.	"	14	2.6	1.08	7	27	22
	Nov. 22, 1934	J. B.	M.	9	2.7	1.23	9	33	20
	Oct. 6, 1936	E. G.	"	7	3.4	1.41	8	25	23
	Average				2.5	1.06	7	28	22
Treated by splenectomy	Dec. 10, 1934	L. L.	"	10	2.8	1.24	6	23	21
	Jan. 31, 1935	R. B.	F.	11	2.2	1.05	7	34	20
	Average				2.5	1.15	7	29	20
Without anemia	Mar. 28, 1935	Z. W.	M.	2	4.7	1.24	12	25	35
	" 26, 1935	C. W.	"	4	4.6	1.18	13	28	36
	Apr. 17, 1935	N. B.	"	6	5.2	1.11	15	29	43
	Average				4.9	1.18	13	27	38
Erythroblastic	Feb. 10, 1936	P. Z.	"	9†	2.4	1.08	6	23	21
	Dec. 13, 1935	"	"	7†	2.6	1.23	5	19	19
	Average				2.5	1.15	5	21	20
Treated by splenectomy	Nov. 26, 1934	M. M.	F.	9	2.9	0.94	6	21	28
	May 2, 1935	"	"	9	3.1	1.10	8	26	26
	Average				3.0	1.02	7	23	27

* See foot-note 1.   † Age measured in months.

Hemolytic and Hypochromic Anemias of Childhood

anemia; *i.e.*, red cell count and hemoglobin.

Volume	Weight	Diameter	Thickness	Diameter to thickness ratio	Volume to thickness index	Resistance				Remarks
						Hypotonic NaCl		Saponin		
						Begin-ning	Com-plete	Begin-ning	Com-plete	
c. μ	micro-micro-grams	μ	μ			per cent	per cent	micro-grams	micro-grams	
86	94	7.2	2.1	3.4	1.17	0.41	0.27	10	43	
94	105	8.6	1.7	5.0	0.76	0.50	0.16	10	50	Sickling moderate, holly leaf
112	123					0.32	0.10	12	33	forms, elevated white, low
89	97	8.2	1.7	4.8	0.83	0.32	0.09	12	40	polymorphonuclear
90	94	8.1	1.8	4.8	0.86					Sickling rapid and extreme,
92	100	7.9	2.0	4.0	1.16	0.38	0.22			crises very severe, low poly-morphonuclear
100	109					0.30	0.08	-		Sickling rapid, high white, low polymorphonuclear
85	92					0.32	0.08	11	67	" "
75	81					0.42	0.10	9	33	Sickling extreme, high white, low polymorphonuclear
68	71	8.3	1.3	6.3	0.61					Sickling rapid, high white
89	97	8.2	1.7	5.0	0.84	0.37	0.10	11	45	
74	81					0.44	0.08	14	40	Sickling slow, splenectomy Feb. 2, 1934
88	95					0.28	0.04	11	33	Sickling rapid, splenectomy Dec. 8, 1926
80	87					0.36	0.06	12	37	
74	81					0.38	0.20	13	59	Sickling marked, white and differential normal
78	85					0.38	0.22	11	50	" "
83	90					0.36	0.22	11	40	Sickling moderate, white and differential normal
78	85					0.37	0.21	11	50	
85	93	7.8	1.8	4.3	0.91					Normal white, low polymorpho-nuclear
75	82	7.6	1.6	4.8	0.87	0.50	0.16			Normoblasts, marked budding of red cell
80	87	7.7	1.7	4.5	0.89	0.50	0.16			
98	107					0.34	0.06	11	50	Splenectomy July 11, 1930, very high white, low polymorpho-nuclear
83	91	9.0	1.3	6.8	0.58	0.32	0.02	15	50	Normoblasts
91	99	9.0	1.3	6.8	0.58	0.33	0.04	13	50	

TABLE II

Observation	Date	Subject	Sex	Age	Red blood cells		Hemoglobin		Hematocrit
					Whole blood	Per gm. cells	Whole blood	Single cells	
				yrs.	millions per c.mm.	$\times 10^{10}$	gm. per 100 cc.	micro-micro-grams	per cent
Hemolytic icterus Severe	Apr. 13, 1936	H. N.	F.	6	2.2	0.85	7	34	24
	May 22, 1936	"	"	6	3.3	1.00	9	29	30
	Dec. 20, 1934	R. F.	M.	4	2.9	0.93	6	22	28
	Average				**2.5**	**0.93**	**7**	**28**	**27**
Mild jaundice	Jan. 21, 1935	E. T.	F.	8	4.0	1.15	13	31	32
	Dec. 13, 1934	C. T.	"	12	4.7	1.25	11	24	34
	Average				**4.3**	**1.20**	**12**	**27**	**33**
Anemia cured by splenectomy‡	Mar. 21, 1935	D. W.	"	12	5.6	1.26	13	22	41
	" 15, 1935	E. H.	"	8	5.9	1.23	15	25	44
	Jan. 16, 1935	P. H.	"	12	6.6	1.28	16	24	48
	Average				**6.0**	**1.26**	**14**	**24**	**44**
Hypochromic Before therapy	Feb. 2, 1935	G. S.	M.	7	3.6	1.16	6	15	28
	Mar. 23, 1935	L. C.	"	2	4.0	1.48	6	14	25
	May 13, 1935	Ro.T.	"	9†	4.6	1.57	7	14	27
	" 20, 1935	Ri.T.	"	9†	5.8	1.81	8	13	29
	Average				**4.5**	**1.51**	**6**	**14**	**27**
After therapy	Mar. 11, 1935	G. S.	"	7	5.4	1.15	11	20	43
	May 27, 1935	L. C.	"	2	5.9	1.39	11	18	39
	June 25, 1935	Ro.T.	"	10†	6.4	1.48	14	22	40
	July 9, 1935	Ri.T.	"	10†	7.0	1.42	12	17	45
	Average				**6.2**	**1.36**	**12**	**19**	**42**

‡ Cured designates those cases whose health has returned to normal by disappearance abnormalities of the erythrocytes persist.

—Concluded

Corpuscular measurements						Resistance				Remarks
						Hypotonic NaCl		Saponin		
Volume	Weight	Diameter	Thickness	Diameter to thickness ratio	Volume to thickness index	Beginning	Complete	Beginning	Complete	
c. μ	micro-micro-grams	μ	μ			per cent	per cent	micro-grams	micro-grams	
108	117	7.0	2.8	2.5	1.60	0.66	0.28			15% reticulocytes
92	100	6.7	2.6	2.6	1.57	0.58	0.26			Splenectomy Apr. 18, 1936, anemia still severe
98	107	.				0.40	0.22	9	29	White normal, low polymorphonuclear, 15% reticulocytes
99	108	6.9	2.7	2.6	1.59	0.55	0.25	9	29	
80	87					0.60	0.34	4	22	White and differential normal
74	80					0.65	0.31	8	33	" " " "
77	84					0.63	0.33	6	28	
73	79	.				0.50	0.30	11	40	Splenectomy July 12, 1933, white differential normal
75	81					0.46	0.26	12	67	Splenectomy June 21, 1933, white differential normal
72	78				.	0.50	0.26	9	67	Splenectomy Apr. 5, 1933, low polymorphonuclear
73	80					0.49	0.27	11	58	
79	86					0.47	0.22	11	33	White and differential normal
62	67					0.46	0.14	9	50	" normal, low polymorphonuclear
59	64	7.8	1.2	6.5	0.63	0.46	0.18	11	45	Premature twins
51	55	7.7	1.1	7.0	0.56	0.40	0.18	7	29	
63	69	7.7	1.2	6.8	0.60	0.45	0.18	9	39	
80	87					0.46	0.21	9	50	Treatment, iron and ammonium citrate
66	72	8.1	1.3	6.2	0.63	0.40	0.16	8	40	Treatment, jeculin
62	68	7.8	1.3	6.0	0.66	0.42	0.22	10	40	" iron and small doses of copper
64	70	7.8	1.3	6.0	0.69	0.44	0.26	11	50	Treatment, liver extract plus iron
68	74	7.9	1.3	6.1	0.66	0.43	0.21	9	43	

of the anemia and accompanying clinical symptoms, although some of the hematological

Additional hematological observations of the erythrocytes in the anemias included the special studies on sickling and fragmentation. A fresh undiluted preparation of blood was made directly from a finger prick. Within 15 minutes this was examined with the oil immersion lens for sickling. The examination was repeated at the end of 1, 4, 12, and 24 hours. A few blood samples showed sickling within the first 15 minutes, most of them within 1 hour, and all at the end of 12 hours. After 24 hours the percentage of sickled cells was determined, which was over 90 per cent in the majority of cases. There was considerable variation in the shape of the sickled cells. Where sickling was most rapid the majority of the cells were greatly elongated with filaments on the ends, whereas the more slowly sickling bloods showed only moderate elongation of the cells with irregularities suggesting crenation, the so called holly leaf forms.

Studies were made for fragmentation of the red blood cell, a fresh preparation being used. The cells were kept at body temperature by placing the microscope in an incubator while the observations were made. At the same time stained smears were examined and found to show practically the same amount of fragmentation (11).

Subjects—The hematological and physical observations on the blood of the individual subjects with the various types of anemia are presented in Table II. The subjects within each group are arranged according to the severity of the anemia as judged by the red blood cell count and hemoglobin value. The cases of hemolytic anemia chosen for study were known to be representative of their respective type, for a number of the patients had been studied intensively for several years in the Children's Hospital of Michigan. Complete clinical and hematological histories are available in publications from the Anemic Clinic[3] (3, 5, 7, 11). Cured hemolytic icterus designates those cases in which the anemia and clinical symptoms had disappeared after splenectomy. The physical characteristics of the erythrocytes however do not return entirely to normal. Although they attain a normal resistance, they remain appreciably smaller, similar to those in the

[3] Dr. Thomas B. Cooley, Director of the Department of Medicine of the Children's Hospital of Michigan, has given generous encouragement and advice on many of the clinical technicalities involved.

chronic mild jaundiced state, indicating that the cause for the structural abnormalities in the cell itself remains after splenectomy.

The cases of sicklemia without anemia[4] include subjects whose red blood cells possess the unique ability of changing to sickle forms under certain conditions, but who demonstrate no accompanying clinical symptoms of anemia or debility. It must be pointed out that the analyses reported herein represent the potential sickle cell and not the cell after it has undergone sickling. When anemia occurs in sicklemia, in addition to the sickling anomaly, it is manifested by clinical symptoms differentiating it from other anemias (5, 6).

Attempts were made to secure studies upon the same patients throughout the course of the anemia, i.e. when suffering a hemolytic crisis, during the less severe chronic state, and after treatment such as splenectomy. The interrelated hematological and chemical study of hemolytic anemias of childhood has included, over a 3 year period, nine cases of sicklemia with anemia, two of whom had been previously splenectomized, and three of sicklemia unaccompanied by anemia; four studies of erythroblastic anemia, two on a small infant and two on a 9 year-old girl who had a splenectomy 4 years before; and three on severe hemolytic icterus in a crisis, two during mild chronic jaundice, and three after cure of the anemia by previous splenectomy. The subjects with hypochromic anemia were selected from the Anemia Clinic[3] of the hospital and from the Infant Growth Clinic[4] of the Children's Fund of Michigan. Studies were made on a pair of 9 month-old twins and two children before and after therapy, when the red blood cell count and hemoglobin had approached normal values.

Fourteen normal children on whom one or more complete blood lipid, mineral, and hematological observations had been made in connection with metabolic studies in progress in the Research Laboratory of the Children's Fund of Michigan comprise the normal group for this study (1).

[4] Dr. Marsh W. Poole and Dr. Brenton M. Hamil kindly made available for study the cases of sicklemia without anemia and some of the hypochromic anemias through the Infant Growth Clinic of the Children's Fund of Michigan.

Lipids of Plasma—Characteristic changes in certain plasma lipids seem to occur in all of the anemias, although the alterations differ in degree (Table III). The four types of anemia herein considered demonstrate an increase of plasma neutral fat which is only slight in hemolytic icterus, moderate in sickle cell and hypochromic anemia, and extreme in erythroblastic anemia. All but hemolytic icterus show a similar elevation of the total lipid values. Phospholipid and free cholesterol show no definite variation, but a reduction of cholesterol esters in the plasma of sickle cell and erythroblastic anemia appears to be a characteristic change.

Although absolute values of plasma lipids vary normally over a moderate range, the percentage of the lipid fractions on the basis of total fat are relatively constant. Thus the calculation of lipid components in terms of per cent of total lipid accentuates the abnormalities in the lipid composition of the plasma as shown in Chart I. The percentage of total lipid in the form of neutral fat is increased, that of cholesterol ester and phospholipid is reduced, while the relative amount as free cholesterol is not definitely altered. The degree of abnormality which is most extreme in erythroblastic anemia appears dependent on the type of anemia rather than the number of corpuscles. The plasma changes observed in these anemias (12) correspond generally to those reported by investigators on pernicious (13, 14) and secondary anemia (15, 16), even though they are different in etiology, hematology, and response to therapy.

Lipids of Erythrocytes—It has been an accepted idea that the erythrocyte composition remains constant under varying states and therefore few studies are available on the lipid distribution of the red blood cell in normal (1, 16, 17) or pathological conditions (13, 15–17) in humans. In harmony with this accepted view a previous study has demonstrated that the lipid composition of the erythrocytes in normal children is relatively constant in amount and distribution (1). On the other hand, each type of anemia herein described demonstrates significant variations from normal in some of the lipid constituents.

The absolute values (on the basis of mg. per 100 gm. of red blood cells) are given in Table III. In contrast to the changes in plasma, augmented total fat values are due to increased free

cholesterol and phospholipid except in erythroblastic anemia, where the neutral fat is disproportionately higher.   The free cholesterol is elevated in the erythrocytes of hypochromic (even after therapy), sickle cell, and erythroblastic anemias, whereas higher cholesterol esters appear in the cells of hemolytic icterus, sickle cell, and hypochromic anemia before therapy.   In the hemolytic icterus erythrocytes the level of total lipids and phospholipids is definitely low.   However, the phospholipid is raised in hypochromic anemia both before and after therapy, while in only one sample of cells of erythroblastic anemia was it markedly elevated.   Recently Whipple and Bradford (18) have observed slight increases in phospholipid of the cells of several cases of erythroblastic anemia.

Inasmuch as wide variations in size and weight of erythrocytes do occur in these different types of anemia (Table II), values expressed in terms of concentration per unit weight may mask important changes in the individual cell, which may have metabolic or structural significance.   In a crisis of hemolytic icterus there were only 0.93×10^{10} cells per gm. of packed erythrocytes as contrasted with 1.51×10^{10} in hypochromic anemia.   Consequently, calculations were made to determine the lipid composition of an average single cell; results for the corpuscular composition are expressed in terms of $\times 10^{-12}$ mg. in Table III.

As illustrated graphically in Chart II, it appears that the elevated values of lipids per 100 gm. of cells in hypochromic anemia are caused by the larger number of erythrocytes present per unit weight of packed cells, each cell containing as much lipid as a normal cell.   The individual corpuscle suffers reduced volume and deficient hemoglobin but retains its full quota of lipids.

The abnormalities in the cells of hemolytic icterus, however, are apparent in the corpuscular composition, whereas, when the anemia is cured by splenectomy, the lipid concentration shows a slight tendency to return to normal.

The cellular compositions in the other anemias demonstrate abnormalities parallel to those brought out by the values per 100 gm. of cells.

In addition to the absolute concentration of lipids in the erythrocyte, the percentage distribution of the various lipid constituents in the total lipid, which normally maintains a characteristic

TABLE III

Blood Lipids in Hemolytic and Hypochromic Anemias of Childhood

The figures in bold-faced type are averages.

Observation	Subject*	Plasma — Protein (per cent)	Plasma — Total lipid (mg. per 100 cc.)	Plasma — Phospholipid	Plasma — Neutral fat	Plasma — Chol. Total	Plasma — Chol. Free	Plasma — Chol. Esters	Eryth. — Total lipid (mg. per 100 gm.)	Eryth. — Phospholipid	Eryth. — Neutral fat	Eryth. — Chol. Total	Eryth. — Chol. Free	Eryth. — Chol. Esters	Corpuscle — Total lipid ($\times 10^{-12}$ mg. per cell)	Corpuscle — Phospholipid	Corpuscle — Neutral fat	Corpuscle — Chol. Total	Corpuscle — Chol. Free	Corpuscle — Chol. Esters	Remarks
Normal	**16**	**7.1**	**454**	**136**	**100**	**143**	**35**	**182**	**424**	**244**	**51**	**116**	**97**	**32**	**399**	**233**	**44**	**110**	**92**	**30**	
Sicklemia‡ Anemia	C. D.	7.4	393	142	71	124	44	136	414	218	48	144	137	11	434	229	50	151	144	12	Severe crisis
	"	7.9	536	186	151	137	50	147	496	235	0	200	111	150	608	288	0	245	136	184	Very severe
	"	7.9	514	145	183	127	42	145	401	241	0	154	146	14	390	234	0	150	142	14	Severe
	E. D.	7.4	451	95	265	66	30	61	379	219	70	90	90	0	378	218	69	89	89	0	" crisis
	A. B.	8.1	614	156	261	137	49	148	498	274	57	165	162	5	543	299	62	180	176	6	"
	B. D.		429	128	113	126	37	151	513	286	56	151	122	50	474	263	52	139	112	27	"
	J. B.	8.3	443	101	222	84	33	86	868	389	0	351	165	314	706	316	0	285	134	255	Moderate
	L. L.	8.6	709	202	251	176	59	197	596	308	100	157	112	75	479	248	80	126	90	60	Severe, splenectomy Feb. 2, 1934
	R. B.	**7.6**	**380**	**97**	**111**	**116**	**36**	**136**	**442**	**239**	**47**	**149**	**139**	**17**	**422**	**228**	**45**	**142**	**133**	**16**	Severe, splenectomy Dec. 8, 1926
Without anemia	Z. W.	6.7	467	113	144	143	45	165	512	268	42	173	132	69	493	258	40	167	128	66	
	C. W.	6.8	518	140	142	160	51	185	435	235	49	130	100	50	369	204	15	133	110	40	
	N. B.	7.6	660	171	222	182	59	208	484	275	72	138	138	0	435	247	65	124	85	42	
		7.0	**548**	**141**	**169**	**162**	**52**	**186**	**458**	**254**	**47**	**144**	**125**	**32**	**390**	**217**	**40**	**122**	**106**	**27**	

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Diagnosis	Subject	Conc.‡																			Remarks
Erythroblastic	P. Z.		707	117	431	110	40	119	571	283	122	148	123	42	466	231	100	121	101	34	Severe crisis
	"	6.5	440	63	228	102	35	114	518	304	68	146	146	0	481	282	63	135	135	0	"
	M. M.	8.2	787	164	428	139	58	137	910	555	155	200	200	0	970	592	165	213	213	0	Very severe, splenectomy July 11, 1930
		7.8	768	103	527	106	58	80	1396	220	896	280	155	14	1264	199	812	254	150	11	Severe
		7.5	**676**	**112**	**404**	**114**	**48**	**112**	**849**	**341**	**310**	**194**	**156**	**14**	**795**	**326**	**285**	**181**	**150**	**11**	
Hemolytic icterus	H. N.	6.7	477	133	159	123	62	126	287	126	50	88	56	55	336	148	59	103	66	65	Hemolytic crisis
	"	7.0	386	111	93	120	0	232	345	180	76	89	89	0	346	180	76	89	89	0	Severe, splenectomy Apr. 18, 1936
	R. F.	7.4	627	159	221	167	51	196	413	247	15	125	82	72	443	261	16	134	88	77	Severe
	E. T.	7.6	363	81	113	112	30	139	408	200	0	159	89	119	356	174	0	139	77	104	Mild jaundice
	C. T.	7.4	486	154	162	112	37	127	392	205	62	116	102	24	314	164	59	93	82	19	"
		7.2	**468**	**128**	**150**	**127**	**36**	**164**	**369**	**192**	**41**	**115**	**84**	**52**	**359**	**185**	**42**	**112**	**80**	**54**	
Cured by splenectomy	D. W.		509	151	75	186	46	237	450	259	0	161	117	74	356	205	0	127	93	59	No anemia, splenectomy July 12, 1933
	E. H.		531	102	137	192	45	247	484	264	46	150	115	59	394	215	37	122	94	48	No anemia, splenectomy June 21, 1933
	P. H.		451	109	172	116	38	132	307	172	29	106	106	0	240	135	23	83	83	0	No anemia, splenectomy Apr. 5, 1933
			497	**121**	**128**	**165**	**43**	**206**	**414**	**232**	**25**	**139**	**113**	**44**	**330**	**185**	**20**	**111**	**90**	**34**	
Hypochromic, Before therapy	G. S.	8.3	641	151	251	160	47	192	509	300	70	135	129	10	438	258	60	116	111	9	Severe
	L. C.	6.9	369	86	94	129	45	142	592	341	57	178	155	39	399	230	38	120	104	26	Very severe
	Ro. T.	6.7	647	160	207	186	49	231	556	305	27	191	145	79	355	195	17	122	92	50	Severe
	Ri. T.	7.0	786	210	309	179	52	215	505	261	0	210	161	83	279	144	0	116	89	46	"
		7.2	**611**	**152**	**215**	**164**	**48**	**196**	**541**	**302**	**39**	**179**	**148**	**52**	**368**	**207**	**29**	**119**	**99**	**34**	
After therapy	G. S.	7.3	430	101	121	143	48	160	454	304	22	128	128	0	393	263	19	110	110	0	Mild
	L. C.	7.7	303	91	49	115	45	119	543	339	0	190	170	34	390	244	0	136	122	24	"
	Ro. T.	6.5	561	158	124	195	75	204	624	258	138	200	159	41	424	175	94	136	108	47	Normal
	Ri. T.	7.8	752	182	239	219	58	273	500	285	21	178	154	41	351	200	15	125	108	29	"
		7.3	**512**	**133**	**133**	**168**	**57**	**188**	**530**	**297**	**45**	**174**	**153**	**35**	**390**	**221**	**32**	**127**	**112**	**25**	

* The sequence of date, age, and sex is the same as in Table II.

† Represents the concentration in an average single red blood cell.

‡ See foot-note 1.

pattern, is altered in certain of these anemias, as shown in Chart I. The red blood cells in erythroblastic anemia exhibit the most abnormal percentage distribution with the neutral fat portion markedly increased and the phospholipid and ester cholesterol fractions diminished. The greater percentage of cholesterol ester in total lipid of sickle cell anemia and hemolytic icterus erythrocytes (both before and after splenectomy) characterizes them in
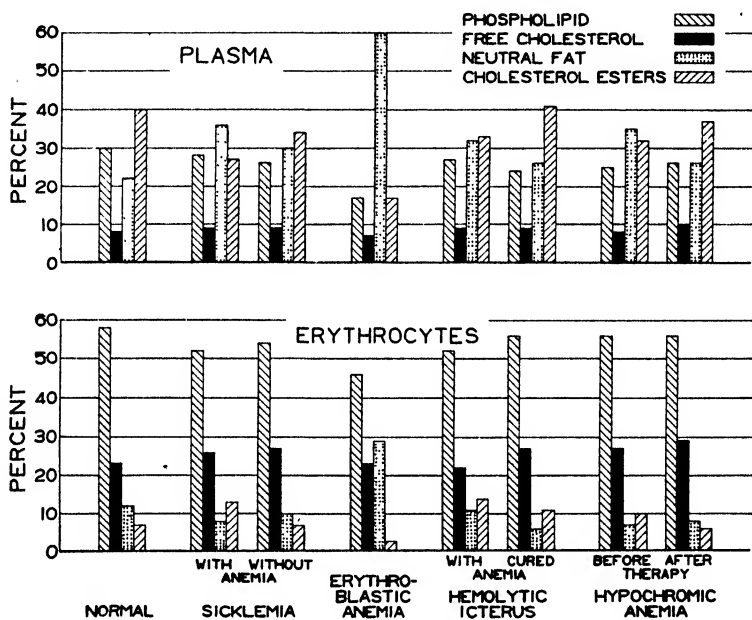
CHART I. Percentage distribution of total lipids in plasma and erythrocytes of hemolytic and hypochromic anemias of childhood.

relation to normal as well as to the other anemias. More of the total lipid in the form of free cholesterol occurs in all but erythroblastic anemia and hemolytic icterus before splenectomy.

Reports in the literature (1, 19–21) have made evident the fact that the amount of cholesterol divided between the free and esterified form in the plasma is normally a physiological constant except in the neonatal period. In the plasma of the different childhood anemias there is a tendency for the percentage cho-

lesterol in the esterified form to be reduced as is shown in Table IV. On the other hand, the percentage of esterified cholesterol in the cells of the anemias is fairly normal except in hemolytic icterus where it is elevated.

The separation of phospholipids into component fractions[5] in the blood of normal children (1) has indicated that the division of
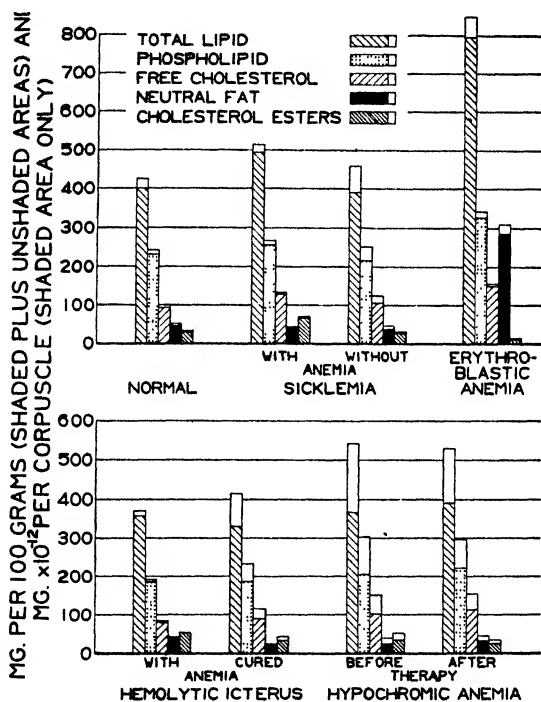
CHART II.   Lipid concentration of erythrocytes in anemias of childhood

the phospholipids may prove to be as physiologically significant as the distribution of total lipids and cholesterol.   The percentage of

[5] In order to check the reliability of this method for the separation of lecithin and cephalin, extensive studies including analysis of other constituents of these fractions are being carried on in this laboratory.   Furthermore, efforts are being made to determine a third fraction of the phospholipids which is indicated to be sphingomyelin by observations made up to the present.

phospholipid as absolute alcohol-soluble in the plasma of the hemolytic anemias, ranging from 90 to 95 per cent, is about the same or slightly higher than that observed in normal children, which averaged 91 per cent (Table IV). It is lower, however, in hypochromic anemia, in which there was an average of 84 per cent phospholipid in the absolute alcohol-soluble form. This fraction, which normally averages 80 per cent in the erythrocyte, is generally lower in the anemias, particularly in the anemia of hemolytic

TABLE IV

Distribution of Cholesterol and Phospholipid Fractions in Hemolytic and Hypochromic Anemias of Childhood

Observation	Plasma			Erythrocytes		
	Phospholipid, alcohol-soluble in total*	Cholesterol, free in total	Phospholipid to free cholesterol ratio	Phospholipid, alcohol-soluble in total*	Cholesterol, free in total	Phospholipid to free cholesterol ratio
	per cent	per cent		per cent	per cent	
Normal.....................	91	24	3.9	80	84	2.5
Sicklemia†						
Anemia..................	95	35	3.3	77	82	2.1
Without anemia..........	93	32	2.7	83	82	2.1
Erythroblastic anemia.....	95	42	2.3	86	94	2.4
Hemolytic icterus						
Anemia..................	90	29	3.6	63	74	2.3
Cured...................	97	27	2.8	90	83	2.0
Hypochromic anemia						
Before therapy...........	84	30	3.1	76	84	1.9
After "	91	34	2.3	73	89	2.2

* Total phospholipid refers to the petroleum ether phospholipid of the "alcohol-ether" extracts of plasma and red blood cells.

† See foot-note 1.

icterus in which only 63 per cent of the phospholipid is absolute alcohol-soluble. In contrast, it is greater than normal in both plasma and cells, 97 and 90 per cent respectively, when splenectomy had resulted in the cure of the anemia of hemolytic icterus.

Minerals—The importance of the blood electrolytes, sodium, potassium, and chloride in respiratory exchange (22) and maintenance of osmotic equilibrium (23) within the body is well established. The abnormal types of erythrocytes in the hemolytic

anemias suggest derangement in both respiration and osmotic equilibrium. For instance sickling is brought about in sicklemia by anoxemia, carbon dioxide, or changes in the acidity of the medium (5, 6). The fact that acidification by variations of carbon dioxide tension and changes in oxygenation of hemoglobin cause both water and chloride to pass into the cells, whereas alkalinization has the opposite effect (23), leads one to suspect that the ionic equilibrium may become upset after sickling. Therefore, it would seem that a study of the electrolyte distribution in the anemias under consideration would be of equal importance to the lipid determinations in contributing to an understanding of the abnormalities occurring in the cells, and in their production and destruction.[6]

The determinations on the sodium, potassium, and chloride distribution between plasma and erythrocytes are given in Table V. Both the range and average chloride content of the serum in all of the anemias are normal. The serum sodium is slightly lower than normal in all of the anemias as well as in cured hemolytic icterus. Potassium values exhibit a wider range of variation than has been observed in our normal studies (1). As in the case of the serum practically all of the erythrocyte chloride determinations fall within the normal range, while the sodium content of the erythrocytes (milli-equivalents per liter of cells) appears to be unstable, as shown by the occurrence of values both higher and lower than normal in the same type of anemia. The potassium of the red blood cells in erythroblastic anemia and severe hemolytic icterus exceeds the normal maximum, while some of the sickle cell values fall below the normal minimum. In certain of these anemias calculation of the mineral constituents of the individual corpuscle, as has been previously pointed out in the discussion of the lipid composition, presents abnormalities obscured in the mineral determinations on a composite basis. The greatest difference between corpuscular and unit weight compositions is shown in hypochromic anemia, in which potassium and chloride

[6] The authors appreciate the helpful contributions of Dr. Hugo A. Freund and his suggestion for including the observations on the mineral distribution of the cells and plasma. Unfortunately it has not been possible to include the mineral determinations at all times because of the limited quantity in the blood samples.

TABLE V

Distribution of Sodium, Potassium, and Chloride of Blood in Hemolytic and Hypochromic Anemias of Childhood

Observation	Subject*	Serum			Erythrocytes			Corpuscles†			Remarks
		Sodium	Potassium	Chloride	Sodium	Potassium	Chloride	Sodium	Potassium	Chloride	
		m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	$\times 10^{-13}$ m.-eq.	$\times 10^{-13}$ m.-eq.	$\times 10^{-13}$ m.-eq.	
Normal	18	**142**	**4**	**104**	**16**	**115**	**53**	**14**	**98**	**45**	
Sicklemia‡ Anemia	A. B.	139	8	108	14	106	40	14	106	40	Severe
	B. D.	135	5	101	28	119	51	23	101	43	"
	C. D.	107	3	105	14	95	61	16	107	69	"
	"	140	5	103	0	124	33	0	111	31	"
	E. D.	132		96	12		56	10		51	Crisis
	R. B.	137	4	106	40	85	39	35	74	34	Severe, splenectomy Dec. 8, 1926
		132	**5**	**103**	**18**	**106**	**47**	**17**	**100**	**45**	
No anemia	C. W.	134	3	101	14	91	39	11	70	30	
	Z. W.	135	2	102	13	108	56	10	80	42	
	N. B.	146	4		2	113		2	93		
		138	**3**	**102**	**10**	**104**	**48**	**8**	**81**	**34**	
Erythroblastic Anemia	M. M.	138	4	110	21	150	46	17	127	38	Severe, splenectomy July 11, 1930
	P. Z.	135	4	101	90	142	50	76	121	43	Crisis
		137	**4**	**106**	**55**	**146**	**48**	**47**	**123**	**41**	
Hemolytic icterus Anemia	H. N.	**136**	**4**	**103**	**22**	**165**	**52**	**20**	**151**	**48**	Splenectomy June 21, 1933
	E. H.	131	3	99	1	111	48	1	83	36	" July 12, 1933
Cured	D. W.	137	4	108	16	112	41	12	81	30	
		134	**4**	**103**	**9**	**111**	**45**	**7**	**82**	**33**	

Hypochromic Anemia										
Before therapy	L. C.	127	8	100	25	104	59	15	64	37	Severe
	G. S.	132	4	106	21	145	53	17	114	42	"
	Ri.T.	136	5	105	0	133	51	0	68	26	"
	133	**6**	**104**	**15**	**129**	**54**	**11**	**82**	**35**		
After therapy	L. C.	**132**	**4**	**105**	**49**	**135**	**55**	**33**	**89**	**36**	Slightly subnormal

The figures in bold-faced type are averages except where preceded by a subject designation, in which case a distinct classification within the group is indicated, although reported by a single case.

* The sequence of date, age, and sex is the same as in Table II.

† Represents the concentration in an average single red blood cell.

‡ See foot-note 1.

values of the individual red blood cell are significantly below normal.   The corpuscular composition emphasizes the elevated potassium in the erythroblastic and hemolytic icterus cells and lower values for all three of the minerals in the cells of sicklemia and clinically cured hemolytic icterus (without anemia).

Maizels (24) has shown in his paper on the ion content of blood in anemias that the amount of base present in the erythrocyte is greater than that required to combine with cell chloride, bicarbonate, and hemoglobin, which is suggested to be in combination with some other unknown anion (X^-) present in the cell.   In the normal erythrocyte of adults excess base values ranged from 7 to 20 milli-equivalents per liter of cells.   In the erythrocytes of most of the anemias except hemolytic icterus the amount of additional cations (potassium and sodium) and therefore the anion (X^-) was found to be increased.

Similar calculations have been made with the present data, in which the results are expressed in milli-equivalents per liter of cells and $\times 10^{-13}$ milli-equivalent per single cell.   It was necessary to use the data of Maizels for the concentration of bicarbonate in cells, but inasmuch as his values remained fairly constant, the use of the average concentration in our calculations should incur little error.   The average normal excess base is approximately 15 milli-equivalents per liter and 13×10^{-13} milli-equivalent per cell (Table VI).   The excess of cations in erythroblastic cells, approximately 94 milli-equivalents per liter or 79×10^{-13} milli-equivalent per cell, exceeds any value reported by Maizels (24) or observed in any of the other groups in this study.

A definite parallelism seems to exist between the excess corpuscular base and the undetermined weight of the cell, which is calculated by subtracting the sum of the corpuscular hemoglobin, water, total ions, and total lipids from the corpuscular weight. Approximately 3 micromicrograms remained undetermined in the normal red blood cell and approximately 8 in the cells of anemia, except in sickle cell anemia in which only 1 micromicrogram remained unaccounted for.   The undetermined weight is probably composed chiefly of stroma protein in addition to other substances such as non-protein nitrogen compounds, phosphoric esters (23, 25), and of nuclear material in the nucleated cell.   Thus the presence of many nucleated cells in the anemias, particularly of the

TABLE VI

Comparison of Total Cation and Anion Content of Erythrocyte in Hemolytic and Hypochromic Anemias of Childhood

Observation	Cations			Anions				Cations minus anions	Excess cations per cell	Undetermined cell weight‡
	Sodium	Potassium	Total	Chloride	Bicarbonate*	Hemoglobin†	Total			
	m.-eq. per l. cells	m.-eq. per l. cells	m.-eq. per l. cells	m.-eq. per l. cells	l. cells	m.-eq. per l. cells	m.-eq. per l. cells	m.-eq. per l. cells	$\times 10^{-13}$ m.-eq.	$\times 10^{-13}$ gm.
Normal..................	17	115	132	53	15	49	117	15	13	3
Sicklemia§										
With anemia...........	18	106	124	47	15	50	112	12	11	2
Without anemia........	10	104	114	48	15	52	115	-1	-2	1
Erythroblastic anemia...	55	146	201	48	15	44	107	94	79	8
Hemolytic icterus										
With anemia...........	22	165	187	52	15	48	115	72	66	7
Cured.................	9	111	120	45	15	44	104	16	12	3
Hypochromic anemia										
Before therapy........	15	129	144	54	15	34	103	41	25	8
After therapy.........	49	135	184	55	15	41	111	73	48	6

* The milli-equivalents of bicarbonate were taken as the average of values found by Maizels in anemia (24).
† The base combined with hemoglobin calculated on the basis of 45 milli-equivalents of base bound by hemoglobin per liter of cells in normal blood ((23) p. 99).
‡ Corpuscular weight minus corpuscular concentrations of (hemoglobin + water + total lipids + total ions).
§ See foot-note 1.

erythroblasts in erythroblastic anemia, may bear some relation to the increased excess cation content and undetermined weight.

Physicochemical—An increased fragility to saponin and hypotonic NaCl is one of the cardinal characteristics of the more spherical erythrocyte in hemolytic icterus (3, 4, 26), whereas cells less vulnerable than normal predominate in erythroblastic anemia (7-9, 26) and sicklemia with and without anemia (5, 7, 26). These characteristics are illustrated by the resistance studies on the red blood cells, as shown in Table II.

A variety of factors such as age (27), chemical composition, particularly proteins (28), lipids (29, 30), and electrolytes (25, 31–34), as well as maturity (35, 36), volume (37), and size and shape (38) of the cell itself have been suggested as important in regulating the resistance of erythrocytes to hemolysis. In the present study there appears to be no definite relationship between the protein content of plasma or cells, the corpuscular volume, cell hemoglobin, or reticulocyte count and hemolysis by hypotonic sodium chloride and saponin solutions.

The present lipid studies give evidence contrary to the widespread theory (29, 30) that the relative proportion of phospholipid and cholesterol in the erythrocyte regulates its resistance to hemolysis, and agree with those of Ponder (25) and recent reviews on phospholipids (39) and cholesterol (40) in that this relationship has been overemphasized without experimental proof. The phospholipid to cholesterol ratio of the fragile hemolytic icterus cell is about the same as that of the average normal red blood cell and the more resistant cells in erythroblastic and sickle cell anemia, as shown in Table II. Furthermore, the ratio of these constituents is more constant in the plasma and shows no relation to the resistance of the erythrocyte.

Permeability studies have yielded conflicting conclusions regarding the relation of the ion content of the erythrocyte, its surrounding medium, and resistance to hemolysis. Ashby (31) has pointed out that a marked inverse parallelism exists between the potassium content of the red blood cell (in which potassium is the predominating cation) and its resistance to hypotonic sodium chloride. Brooks (34), on the other hand, found no definite change in the fragility of potassium-rich and potassium-poor cells. In the present study the highest potassium values observed occurred in erythroblastic anemia with 123×10^{-13} milli-equiva-

lent per cell and in hemolytic icterus with 151×10^{-13} milliequivalent per cell. Inasmuch as these anemias are characterized by erythrocytes diametrical in their resistance to hemolysis, there seems to be no relationship between fragility and potassium content. Although Maizels (24) has reported a low potassium content of the cell in hemolytic icterus, he did not observe any relation between the level of potassium content of the cell and its fragility or destruction. The present data show no apparent relationship of the sodium, chloride, or total cation content of either cells or serum to the fragility of the cells in the different types of anemia.

Relation of Red Blood Cell Composition to Physical Properties— Haden (38) has pointed out through a study of the dimensions of the cell, including volume, diameter, and thickness, that the shape of the erythrocyte influences its hemolysis by hypotonic sodium chloride. He has shown that the fragile hemolytic erythrocyte is more spherical than normal with a diameter to thickness ratio of 2.1, and a volume to thickness index of 1.92 contrasted with a diameter to thickness ratio of 4.1 and volume to thickness index of 1.00 in a normal erythrocyte. This fact is corroborated by the data on hemolytic icterus herein recorded (Table II). In addition the data on sickle cell and erythroblastic anemias with cells more resistant to hemolysis by hypotonic sodium chloride demonstrate thinner and more discoidal shapes, as shown by average diameter to thickness ratios of 5.0 and 5.7 and volume to thickness indices of 0.84 and 0.74 respectively. This relationship is further substantiated by the increased resistance to hemolytic agents (41) of the eliptical red blood cell which anomalously predominates in the blood of certain individuals (41, 42). On the other hand, the small cell of hypochromic anemia seems to be less resistant to osmotic hemolysis than its discoidal shape would indicate, the diameter to thickness ratio and the volume to thickness index being 6.8 and 0.60 respectively. Its cell area, however, is practically normal as compared with the increased areas of the flattened erythrocytes of sickle cell and erythroblastic anemias.[7]

[7] The formula of Emmons (43), area = $2\pi r(r + h)$, in which the cell is considered as a flat cylinder, r being half the cell diameter and h the cell thickness, was used to calculate the cell areas in our study. Although it has been pointed out that this formula gives results which are consistently too high (25), it seems to yield relative values for comparative purposes.

Although hemolysis by saponin involves a different mechanism from that of hypotonic NaCl in that it appears to combine with certain constituents of the red blood cell itself, this process likewise seems to be affected by the shape of the cell, except in hypochromic anemia, as shown by the data in Table II.

Ponder ((25) p. 72) has described the effect of saponin which is typical of most lysins on the shape of the red blood cell as follows:

"At first the cells show no noteworthy change; there may be some crenation, but sometimes there is none, and any that there is usually tends to become less as time goes on. Just before hæmolysing, however, each cell becomes perfectly spherical; it soon afterwards loses its pigment, and fades from view. The change from the discoidal to the spherical form does not seem to involve any very noticeable alteration in volume; one gets the impression that the 'structure' which maintains the flattened form of the cell suddenly gives way, and that the sudden removal of restraint is rapidly followed by lysis."

Therefore the change of the more discoidal cells to the spherical form before hemolysis seems to require more drastic hemolytic action. The cells in sicklemia behave anomalously from this point of view. After the potential or latent sickle cell, which is more resistant than the normal cell, has once undergone sickling, becoming thinner and even more discoidal, it seems to become more susceptible to hemolytic influences, a fact that has been demonstrated *in vitro* by the increased hemolysis and ultimate disappearance of the sickle-shaped cells (5, 44).

It is evident from the foregoing observations that the shape of the red blood cell, as expressed by volume, diameter, and thickness relationships, plays a significant rôle in its physicochemical behavior. Moreover, that the physical contour of the erythrocyte is dependent upon its chemical composition, and that of its surrounding medium, is indicated by experiments on the change in shape under the influence of certain factors. For instance, cells become spherical when suspended in isotonic sodium chloride solution between two glass surfaces, a change which will not occur if plasma is present ((25) p. 75); addition of lecithin converts cells to the spherical form, even in the presence of plasma ((25) p. 81); and in sicklemia the contour of the cell appears to be related to the state of combination of hemoglobin, since sickling is brought about *in vitro* by anoxemia or by a change in the acidity of the medium (5, 6, 44, 45).

An examination of the present data has failed to disclose any relationship of the lipid or mineral content or distribution in either the plasma or erythrocytes to the characteristic abnormal shape, size, or resistance of the cells in the anemias under consideration. There are slight indications that there may be some relationship between the cell area and concentration of lipids. Why the erythrocytes in certain types of anemia do not retain the normal biconcave shape which Ponder (46) has shown to be the most efficient form for the rapid diffusion of gases remains to be determined.

Comment

The pathological changes which the corpuscle undergoes in these different anemias may result in an impairment of its physiological activity or efficiency. On the other hand, the individual erythrocyte in the anemias may be more active because of increased demands placed upon the reduced numbers to carry on the normal hematological and metabolic functions. The influence of the maturity of the cell is an additional factor to be considered. The presence of large numbers of reticulocytes in hemolytic icterus and of immature erythroblasts in erythroblastic anemia would appear to alter the chemical picture if they differ in activity and composition from the older mature forms. It is well established that reticulocytes have an augmented metabolism as measured by rate of respiration (36), whereas erythroblasts appear to have a much higher oxidative and glycolytic metabolism comparable to that of embryonic tissue (47).

Physiological activity is not confined to respiratory exchange, a fact emphasized by Bloor (48) in the following statement.

"The term 'physiological activities' as used here is thus meant to include all the processes of the living cell. Of these, oxidation is the basic one, upon which the others depend to the extent at least of their energy requirement, and . . . oxidation is the function most emphasized in discussions of the mitochondria and the phospholipids. But it should be borne in mind that it is only one form of activity and perhaps not the most important except in muscle where energy transformations are quantitatively the most significant. Yet the muscles (except the heart) have the lowest phospholipid and cholesterol content of all the tissues. In other tissues such as the brain, liver, and kidney having a much higher lipid content, energy transformations are relatively less important and other activities than oxidation must obviously bear greater significance in the consideration of the total physiological activity of most cells."

The lipid and mineral content of tissues in relation to their physiological activity or efficiency has been the subject of numerous investigations. Bloor and his school (48–50) have demonstrated that increased activity is associated with higher values of phospholipid and free cholesterol, whereas lowered physiological activity, degeneration, and retrogression are accompanied by lower levels of these two lipids together with increased amounts of neutral fat and cholesterol esters. In regard to the minerals, Gérard (51) has concluded from a study of the potassium-sodium content of tissues that those of most active function, whether glandular, nervous, or muscular, have the highest potassium to sodium ratio.

The abnormalities which occur in the lipid and mineral composition of the erythrocytes in the congenital anemias of childhood may be of significance in the light of these theories of physiological activity.

Outstanding are the cells in erythroblastic anemia in which there is a relatively large amount of neutral fat present, thus signifying that the erythrocyte is less active. Furthermore, the relative percentage increase in the cholesterol ester of the cell in sickle cell anemia suggests degeneration and retrogression. On the other hand, the percentage lipid distribution is normal in the sicklemia cell without anemia and it would appear therefore that the changes indicating retrogression are probably associated with the anemia rather than with the phenomenal ability of the erythrocyte to sickle. The erythrocyte of hemolytic icterus with severe anemia has relatively less phospholipid and more cholesterol ester than normal, thus showing signs of diminished activity and degeneration. After splenectomy, which cures the anemia, the relative proportions of the different lipid fractions in the cells become more normal. Finally, we observe in hypochromic anemia, a normal pattern of lipid distribution both in the anemic state and after the improvement of the red blood cell count by therapy.

The potassium to sodium ratio in addition to the lipid composition furnishes confirmatory evidence that the physiological activity of the erythrocyte is diminished in the hemolytic anemias. This ratio is 2.6 in erythroblastic anemia as compared with 7.0 in cells of normal children. In sickle cell anemia it is 5.9, but in sicklemia

without anemia the ratio is found to be 10.4.   Before and after
splenectomy in hemolytic icterus the potassium to sodium ratio is
6.5 and 9.9 respectively.

To test further the application of the theory that a lowered
potassium to sodium ratio and diminished physiological activity
are found in the erythrocyte in anemia, ratios have been calculated
from Maizels' extensive data on the anemias of adults (24).
Values lower than normal occur in his cases of macrocytic anemia
and hemolytic icterus with ratios of 7.9 and 6.9 respectively, while
normal ratios (8.8) were found in his cases of microcytic anemia.
In microcytic anemias (hypochromic) the ratios are not found to
be lowered in either adults or children, with values of 8.8 and 8.4
respectively.   It appears therefore with the exception of hypo-
chromic anemias that the potassium to sodium ratio of the erythro-
cyte is decreased in anemia.

These data indicate a lowered physiological activity of the eryth-
rocyte in the hemolytic anemias as compared to the normal red
blood corpuscle, if one accepts the hypotheses that the lipid com-
position and potassium to sodium ratios in tissues are associated
with physiological activity.

A deficient erythrocyte structure, as indicated by an upset lipid
and mineral composition, agrees with the theory advanced by
Cooley (2) on the results of splenectomy in these three types.   His
theory assumes that these cells are abnormal in structure, and
that if their destruction is minimized by splenectomy, the patient
is benefited only in so far as the cells left in circulation are capable
of functioning in a normal way.   Inasmuch as the erythrocytes
are products of the hematopoietic system, the abnormalities in
their structure and composition which persist after splenectomy
give evidence that they may reflect the pathological state and
function of the blood-forming tissue.

In efforts to elucidate further the chemical structure of the red
blood cell, research is being carried on in an attempt to isolate the
"stroma" in a pure state sufficient for detailed chemical analysis.[8]
When one can differentiate between the constituents of the cell
which are bound as the structural framework and those which may
be more mobile or metabolic, one can hope to gain further informa-

[8] Unpublished data; preliminary report (52).

tion of its rôle in metabolism and the significance of its chemical abnormalities in blood dyscrasias.

SUMMARY

Simultaneous analyses of the lipids and minerals in the serum and cells, together with comprehensive hematological and certain physical observations on erythrocytes such as cell volume, weight, diameter, thickness, specific gravity, water content, and resistance to hemolysis against saponin and hypotonic sodium chloride solutions have been made in the hemolytic (erythroblastic, sickle cell, and hemolytic icterus) and hypochromic anemias of childhood. Inasmuch as these blood dyscrasias are characterized by extreme abnormalities in shape, size, weight, and fragility of the red blood corpuscles, the importance of expressing the chemical composition of the erythrocytes on the basis of an average single corpuscle rather than in terms of concentration per unit weight or volume has been emphasized.

The most characteristic change in the plasma lipids occurred in the neutral fat fraction.   There was a marked elevation in erythroblastic anemia with lesser increases in the other types of anemia. The concentrations of the minerals in the serum were within the normal range for chloride, low for sodium, and more variable than normal for potassium.

Abnormalities in the concentration and distribution of the lipids and minerals occur in the erythrocytes which seem to be dependent on the type of anemia and related in some way to the characteristic red blood cell typical of the anemia.

The relation of the free to total cholesterol, the absolute alcohol-soluble phospholipid to total phospholipid, the ratio of cholesterol to phospholipid in regard to hemolysis, and the excess cation concentration in the corpuscles have been discussed with respect to their fluctuations in these diverse anemias.

Measurement of cell volume, diameter, and thickness indicate that the cells of erythroblastic, sickle cell, and hypochromic anemia are more discoidal than normal, whereas those of hemolytic icterus are more spherical.

A study of the resistance of the different types of erythrocytes to hemolysis by saponin and hypotonic sodium chloride solutions in relation to the physicochemical characteristics of the cells has

shown that a correlation seems to exist between the shape and fragility of the corpuscle in the hemolytic anemias.

The pathological changes occurring in the erythrocytes in these various types of anemia together with the alterations in lipid composition and the potassium to sodium ratio have been discussed in the light of the theory of their association with the physiological activity or efficiency.

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THE LIPID AND MINERAL DISTRIBUTION OF THE SERUM AND ERYTHROCYTES IN PERNICIOUS ANEMIA*

BEFORE AND AFTER THERAPY

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It is generally agreed that the abnormally low percentage of red blood cells in pernicious anemia is the result of inhibited erythropoiesis, rather than excessive erythrocyte destruction as was once thought. The investigations of Castle and coworkers (1) have placed this blood dyscrasia, which is caused by the lack of a substance essential for the normal maturation of the erythrocyte, in the category of deficiency diseases. Furthermore, they have established that this condition involves a substance stored in the liver which has been formed through the interaction of an extrinsic factor supplied by the food and an intrinsic factor secreted in the stomach (2, 3). Whipple (4) has called this active principle a "stroma-producing factor," but because injections of small quantities of this substance produce a rise in the blood count of several million red cells, Davidson and Leitch (5) suggest that it is probably of the nature of a hormone or enzyme. Regardless of the nature of the antianemic principle, it appears to be in some way concerned with the formation of the stroma of the red blood cell rather than with the synthesis of hemoglobin (6).

Inasmuch as the erythrocyte in pernicious anemia is grossly abnormal in size and shape (hyperchromic macrocyte) with indications of a deficient chemical structure, and since specific treatment alleviates this pathological condition of the cell, a study of the chemical composition of the cells and plasma in pernicious anemia before and after treatment may yield not only information concerning the defects in structure and physiological activity of the erythrocytes and parent hematopoietic tissue in this disease, but may offer an insight into the structure of cells in general. As a part of a general chemical study (7–10) on the structure of the red blood cell, this report presents hematological observations together with simultaneous determinations of the lipid and mineral (sodium, potassium, and chloride) distribution in the red cells and plasma in pernicious anemia, before and after therapeusis. The lipid and mineral composition was selected for study because certain of these constituents are essential in cellular structure and function (11, 12). The occurrence of neurological symptoms (3, 13) in pernicious anemia and the results from previous studies on the plasma (14, 15) suggest a derangement in fat metabolism, while the constant association of achlorhydria with this disease (16, 17) indicates abnormalities in mineral metabolism.

Subjects—One or more studies (twenty in all) have been made on eight patients. All of the subjects with the exception of St[1] were made available through the facilities of the Anemia Clinic of Harper Hospital. They were representative of typical pernicious anemia[2] and were under close supervision inasmuch as their response to treatment was being used as the basis of potency tests of marketable liver and ventriculin extracts.[3]

The hematological observations on the venous blood samples of

[1] Dr. Raphael Isaacs of the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan, kindly made patient St available for this study.

[2] Subject Md was not included in the average results because of complications in addition to the anemia and death soon after the initial study.

[3] Samples of the ventriculin and liver extracts which were being tested and used in the treatment of their patients were kindly furnished to us by Dr. E. A. Sharp of Parke, Davis and Company. Lipid analyses showed that the liver extracts were free of lipids, and that the ventriculin contained only negligible amounts of lipid, chiefly as neutral fat (approximately 1 mg. per capsule).

the patients in relapse and at intervals after treatment are given in Table I together with pertinent data of age, sex, and type of therapy.   The red blood cell count, hemoglobin, and corpuscular measurements display the typical hematological characteristics of pernicious anemia and the shift toward a normal blood picture[4] with treatment.

In order to simplify discussion the studies have been averaged *arbitrarily* on the basis of red blood cell count and hematological characteristics.   Those in *relapse* include the initial observations made at the time of hospitalization when the red cell counts were not above 2.5 millions per c.mm. and before any treatment had been introduced.   In the course of therapy the cases were considered in *remission* when the red cell counts were approximately 4.5 millions per c.mm. or above with normal hemoglobin levels and corpuscular volume practically normal, and in *partial remission* when the values were intermediate.

Methods

Postabsorptive blood samples (30 to 40 cc.) were obtained from the arm vein for each study, and the following methods were used.

Sodium was determined by the uranyl microgravimetric method ((21) p. 736); potassium by the cobaltinitrite microtitration method ((21) p. 748); and chloride by the open Carius method as applied by Van Slyke and Sendroy ((21) p. 836).   The values for the mineral content of the cells were calculated from the determinations made on the whole blood and the serum.   Nitrogen was determined by the micro-Kjeldahl gasometric technique of Van Slyke ((21) p. 353, (22)).   The lipids were determined by the gasometric procedure of Kirk, Page, and Van Slyke (23). The various methods used in studying the resistance of the erythrocytes have been detailed previously (9).

Corpuscular measurements were as follows: Wintrobe's (24) methods were used in calculating cell volume from the percentage

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[4] Normal values of red blood cell count, hemoglobin, hematocrit, and corpuscular volume are taken from Vaughan (18) and Wintrobe (19); diameter, thickness, and volume to thickness index from Haden (20); resistance to hemolysis in hypotonic sodium chloride solutions from Haden (20) and Vaughan (18); resistance in saponin solutions and remaining calculations from studies in this laboratory (9).

TABLE I

Hematological Observations in Pernicious Anemia before and after Therapy

Date	Subject	Sex	Age yrs.	Red blood cells Whole blood millions per c.mm.	Red blood cells Per gm. cells × 10⁹	Hemoglobin Whole blood gm. per 100 cc.	Hemoglobin Single cell micro-micro-grams	Hematocrit per cent	Corpuscular Volume c.μ	Corpuscular Weight micro-micro-grams	Diameter μ	Thickness μ	Diameter to thickness ratio	Volume to thickness index	Hypotonic NaCl Beginning per cent	Hypotonic NaCl Complete per cent	Saponin Beginning micro-grams	Saponin Complete micro-grams	Reticulocytes per cent	Remarks
Apr. 25, 1935	Md*	F.	69	0.78	0.58	5.0	64	12	147	159	8.2	2.8	2.9	1.37	0.40	0.24	8	29	1.0	Complicated, expired July, 1935
Jan. 2, 1935	St	M.	50+	1.03	0.79	3.1	31	12	117	126	7.9	2.2	3.6	1.20	0.40	0.24	3	33		30 gm. ventriculin daily + 4 cc. liver extract weekly, reticulocyte peak Feb. 26, 1936 (25.6%)
Feb. 8, 1936	Mb	"	31	1.58	0.84	7.5	48	17	110	119	8.0	2.2	3.6	1.09	0.34	0.20				
May 8, 1936				4.60	0.89	13.0	28	48	104	112	7.4	2.4	3.1	1.31					3.1	
Dec. 10, 1935	Lg	F.	63	1.58	0.53	7.5	47	27	173	187	7.8	3.6	2.2	1.87	0.38	0.24			1.7	3 cc. liver extract
" 23, 1935				3.51	0.90	9.9	28	36	103	111	7.7	2.2	3.5	1.14	0.42	0.26			3.2	Reticulocyte peak, Dec. 15, 1935 (9.2%)
Feb. 20, 1936	Sh	"	51	4.49	0.95	13.0	29	44	97	105	7.4	2.3	3.2	1.22						4 cc. liver extract weekly. Fatigue, edema of ankles
May 15, 1936				5.25	1.08	13.0	25	45	85	92	7.2	2.1	3.4	1.15						
June 3, 1935				1.69	0.86	5.5	33	19	110	119	7.9	2.3	3.4	1.13	0.40	0.22	9	33	2.2	3 cc. liver extract weekly, increased to 3 cc. liver extract daily
July 26, 1935				3.88	0.93	11.0	28	38	99	107	8.0	2.0	4.0	0.98	0.38	0.28	11	33		
May 11, 1936				4.47	0.97	12.0	27	42	95	103	7.7	2.1	3.7	1.06					4.9	

Date																			Treatment
Jan. 3, 1936	On	F.	59	1.80	8.5	47	27	150	162	7.7	3.2	2.4	1.67	0.44	0.24			1.8	2 cc. liver extract daily
" 15, 1936				3.38	11.0	33	38	111	120	7.7	2.4	3.2	1.23	0.44	0.22			8.4	Reticulocyte peak Jan. 8, 1936 (14.2%)
May 13, 1936				3.54	11.0	31	38	108	114	7.5	2.4	3.1	1.30						4 cc. liver extract weekly
Feb. 21, 1936				4.14	13.0	31	44	106	115	7.7	2.3	3.3	1.18						3 " " daily
Mar. 17, 1936	Hn	M.	70	1.87	7.0	37	21	110	119	7.9	2.3	3.4	1.13						4 " " twice a wk.
July 28, 1936				3.49	11.0	32	43	122	134	7.6	2.7	2.8	1.41						
May 5, 1936	Sr	"	72	3.63	11.0	30	37	109	119	8.0	2.2	3.6	1.08						3 cc. liver extract twice a wk.
Jan. 24, 1935				2.20	4.5	20	25	113	122	8.1	2.2	3.7	1.08	0.38	0.26	8	33	1.8	10 gm. ventriculin daily, increased to 30 gm.; Mar. 28, 1935 changed to 2 cc. liver extract daily
Apr. 4, 1935				5.90	13.5	23	48	81	88	7.6	1.8	4.2	0.94	0.40	0.26	10	40	3.7	

Averages

Relapse (r.b.c. less than 2.5 millions per c.mm.)	1.68	6.2	38	21	126	136	7.9	2.6	3.2	1.31	0.39	0.23	6	33
Partial remission (r.b.c. 2.5 to 4.5)	3.65	11.1	30	39	108	117	7.7	2.3	3.4	1.19	0.41	0.25	11	33
Remission (r.b.c. more than 4.5)	4.94	12.9	26	45	92	100	7.5	2.1	3.5	1.14	0.40	0.26	10	40
Normal† men	5.4	14.5	29	45	84	91	7.7	2.0	4.1	1.00	0.46	0.28	10	43
" women	5.0	13.6	29	44	88	95								

* See foot-note 2.

† See foot-note 4.

volume and red cell count of whole blood, and corpuscular hemo-
globin from the red cell count and hemoglobin.   The weight was
computed from the erythrocyte volume and specific gravity.   The
thickness was determined from cell volume and diameter by means
of the three-dimensional chart adapted from von Boros by Haden
(20), and volume to thickness index as outlined by Haden with
his nomogram adapted from Warburg.   Computations of cor-
puscular concentrations were made from the determined amount
per 100 gm. of cells and the number of cells per unit weight, the
latter value being calculated from the cell weight.   Cell diameter
was measured directly from a dried smear of blood by Bock's
erythrocytometer, a simplified diffraction apparatus described by
Sharp and Schleicher (25).

The specific gravity of the cells was calculated from the values
of the plasma and whole blood as determined by weighing in
pycnometer bottles on the microbalance (26).   Water content
was determined by drying the cells in sand for 48 hours at 85°.

<center>DISCUSSION</center>

Physicochemical—The macrocytic erythrocyte in pernicious
anemia is more spherical than the normal red blood cell, as shown
by the diameter to thickness ratio and volume to thickness
index (Table I).   In addition, there is a parallelism in the degree
of spherocity, the size of the erythrocyte, and the corpuscular
hemoglobin.

Evidence from other types of anemia indicates that cells of
spherical form are more fragile (27), while erythrocytes more dis-
coidal than normal, with the exception of the cells in hypochromic
anemia, are more resistant to hemolytic agents (10).   The erythro-
cytes in pernicious anemia, however, demonstrate a slight increase
of resistance in both beginning and complete hemolysis by hypo-
tonic sodium chloride, a characteristic pointed out by Vaughan
(18) and Daland and Worthley (28); but they seem slightly more
fragile than normal in saponin solution (Table I).   A consideration
of the comparative areas[5] of the erythrocytes, including both

[5] The formula of Emmons (29), area = $2\pi r(r + h)$, in which the cell is
considered as a flat cylinder, r being half the cell diameter and h the cell
thickness, was used to calculate the cell areas in this study.   Although it
has been pointed out that this formula gives results which are consistently
too high (30), it seems to yield relative values for comparative purposes.

hypochromic and pernicious anemias, suggests a relationship between the fragility and the corpuscular area; a more expansive surface of the erythrocytes in pernicious anemia and other anemias appears to be associated with an increased resistance in hypotonic sodium chloride, while the cell in hypochromic anemia, although microcytic, seems to have a normal surface area and resistance (10).

Lipids in Plasma[6]—The blood lipid picture which is found to represent most clinical anemias is characterized by the appearance of an excess of fat with a simultaneous deficiency of phospholipid and cholesterol (33). Similar results are found in the plasma of pernicious anemia in relapse (Table II). In addition, separation of the cholesterol into the free and combined fractions shows that the most consistent and striking abnormality outside of the increased neutral fat is the decreased cholesterol ester. In practically all cases the individuals respond alike to the effect of therapeusis and increased red blood cell count. As the number of erythrocytes increases and the anemia improves, there is a marked elevation in the cholesterol esters and a decrease in the neutral fat. The phospholipid and free cholesterol fractions do not show the same consistent changes, but do exhibit a tendency to increase after treatment. On the basis of the average figures in mg. per 100 cc. for relapse and remission the phospholipid increases with treatment from 119 to 142, the free cholesterol from 33 to 49, and the cholesterol esters from 126 to 204, whereas the neutral fat decreases from 219 to 135. A comparison of these values with those of Page *et al.* (31) shows that even in remission all of the lipid constituents are lower than in normal plasma. With the exception of neutral fat, however, they are tending towards the normal concentrations as the anemia improves. These changes in plasma lipids from relapse to remission are in general agreement with the comprehensive studies of Muller (15) in pernicious anemia on cholesterol, lecithin, and total fatty acids. The percentage of free cholesterol in total cholesterol (32) which is normally considered to be a physiological constant (27 per cent) is high in

[6] Normal plasma values of total lipid, phospholipid, neutral fat, total and free cholesterol, and cholesterol esters are from Page *et al.* (31). The normal value for percentage of free in total cholesterol is from Sperry (32). All other normal values are from studies in this laboratory (9).

TABLE II

Distribution of Lipids in Blood in Pernicious Anemia before and after Therapy

Date	Subject*	R.b.c. (millions per c.mm.)	Plasma: Total lipid (mg. per 100 cc.)	Plasma: Phospholipid (mg. per 100 cc.)	Plasma: Neutral fat (mg. per 100 cc.)	Plasma: Cholesterol Free (mg. per 100 cc.)	Plasma: Cholesterol Esters (mg. per 100 cc.)	Plasma: Cholesterol Total (mg. per 100 cc.)	Plasma: Free in total (per cent)	Plasma: Alcohol-soluble phospholipid (per cent of total)	Plasma: Protein (per cent)	Eryth.: Total lipid (mg. per 100 gm.)	Eryth.: Phospholipid (mg. per 100 gm.)	Eryth.: Neutral fat (mg. per 100 gm.)	Eryth.: Cholesterol Free (mg. per 100 gm.)	Eryth.: Cholesterol Esters (mg. per 100 gm.)	Eryth.: Cholesterol Total (mg. per 100 gm.)	Eryth.: Free in total (per cent)	Eryth.: Alcohol-soluble phospholipid (per cent of total)	Corpuscle†: Total lipid ($\times 10^{-12}$ mg. per cell)	Corpuscle†: Phospholipid ($\times 10^{-12}$ mg. per cell)	Corpuscle†: Neutral fat ($\times 10^{-12}$ mg. per cell)	Corpuscle†: Cholesterol Free ($\times 10^{-12}$ mg. per cell)	Corpuscle†: Cholesterol Esters ($\times 10^{-12}$ mg. per cell)
Apr. 25, 1935	Md‡	0.78	451	58	236	32	125	106	30	100	5.5	494	15	341	52	86	103	51		786	24	543	83	137
Jan. 2, 1935	St	1.03	429	88	317	16	8	21	76	94	7.1	318	169	0	79	70	120	66	72	401	213	0	99	88
Feb. 8, 1936	Mb	1.58	325	59	141	22	103	83	27	90	6.5	274	132	18	91	32	110	83	78	327	157	22	108	39
May 8, 1936		4.60	462	135	122	28	177	132	21	99	9.9	373	235	23	101	15	110	92	81	419	264	26	113	17
Dec. 10, 1935	Lg	1.58	673	182	303	42	146	129	33	87	7.5	364	213	27	39	85	89	44	91	682	399	51	73	159
" 23, 1935		3.51	507	156	125	27	199	145	19	95		373	246	0	87	40	111	78	67	414	273	0	97	46
Feb. 20, 1936		4.49	488	73	146	67	203	187	36	88	7.1	419	250	22	82	65	120	68	78	440	263	23	86	68
May 15, 1936		5.25	591	200	120	48	223	180	27	95	7.5	395	233	41	120	1	121	99	85	365	215	38	112	1
June 3, 1935	Sh	1.69	634	150	284	44	157	137	32	98	7.9	253	57	28	93	75	153	61		302	68	0	110	123
July 26, 1936		3.88	447	129	107	57	154	148	39	88		418	257	28	119	13	127	94	88	448	276	30	128	14
May 11, 1936		4.47	632	174	205	41	213	167	25		7.2	429	245	31	95	58	129	74	56	443	253	32	98	60
Jan. 3, 1936	On	1.80	461	114	171	45	131	122	37	100		322	186	0	61	75	106	58	58	523	302	0	99	122
" 15, 1936		3.38	478	152	146	36	145	121	30	81	7.5	355	262	0	93	0	93	100		426	315	0	112	0
May 13, 1936		3.54	425	154	83	36	152	126	29	97	6.9	415	241	52	86	36	107	80	72	474	275	59	98	41
Feb. 21, 1936		4.14	596	166	150	2	278	166	1	92	7.2	414	252	15	59	88	111	53	69	477	290	17	68	101

Mar. 17, 1936	Hn	1.87	347	109	102	24	112	76	32	91	6.4	331	209	0	55	67	94	59	61	393	248	0	65	80
July 28, 1936		3.49	419	85	161	12	160	107	11	94	8.0	401	247	18	59	77	104	57	74	535	330	24	79	103
May 5, 1936		3.63	364	112	64	35	154	126	28	98	7.0	354	189	31	81	54	113	72	89	420	224	37	96	64
Jan. 24, 1935	Sr	2.20	607	134	213	36	224	169	21	98	7.7	300	106	53	91	51	121	75	61	367	129	65	112	62
Apr. 4, 1935		5.90	470	129	81	59	202	178	33	95	8.3	415	253	29	110	24	124	89	79	365	222	25	97	21
Averages																								
Relapse (r.b.c. less than 2.5 millions per c.mm.)			497	119	219	33	126	105	37	94	7.2	309	153	14	73	69	113	64	70	414	217	20	95	96
Partial remission (r.b.c. 2.5 to 4.5)			462	136	119	29	177	134	22	92	7.3	390	242	21	83	44	109	76	74	456	283	24	97	53
Remission (r.b.c. more than 4.5)			529	142	135	49	204	204	28	94	8.0	406	243	29	102	33	121	84	81	406	243	29	101	33
Normal§			735	181	225	82	254	232	27	91	7.1	424	244	51	97	32	116	84	80	399	233	44	92	30

* The sequence of date, age, and sex is the same as in Table I.

† Represents the concentration in an average single red blood cell.

‡ See foot-note 2.

§ See foot-note 6.

relapse (37 per cent), but normal after remission of the anemia (28 per cent).

The absolute concentration of the different lipids may vary normally over a wide range, but the percentage of these fractions in the total lipid tends to be quite uniform. The composition of the lipid mixture in the plasma in pernicious anemia before and after treatment is shown graphically in Chart I. These values are calculated from the average figures given in Table II. In the
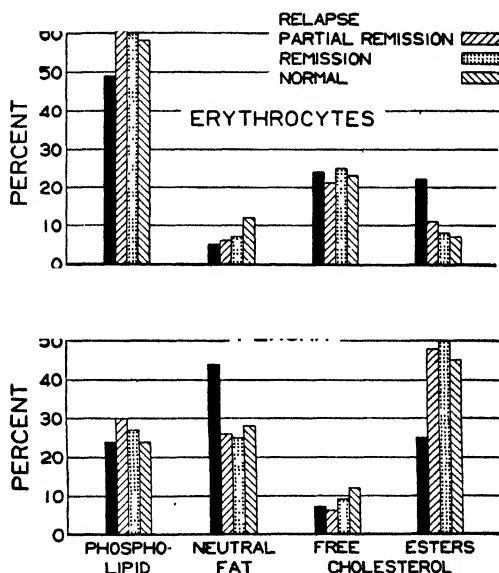
CHART I. Percentage composition of total lipids of plasma and erythrocytes in pernicious anemia.

plasma, the striking change is the increase of cholesterol esters after therapeusis, with a corresponding decrease in neutral fat. This reciprocal relationship of cholesterol esters and neutral fat has been pointed out by Page *et al.* (31) in their study of the lipids of normal blood plasma. The percentage of total lipid as free cholesterol is increased with remission but is still less than normal. On the contrary, the percentage phospholipid is normal in relapse and increased after treatment.

Lipids in Erythrocytes—Few workers have been concerned with

the lipid changes in the erythrocytes themselves, inasmuch as it was early reported (14) that the corpuscles in anemia as well as in other conditions tend to preserve a constant composition, abnormalities being found mainly in the plasma. Bodansky (34),
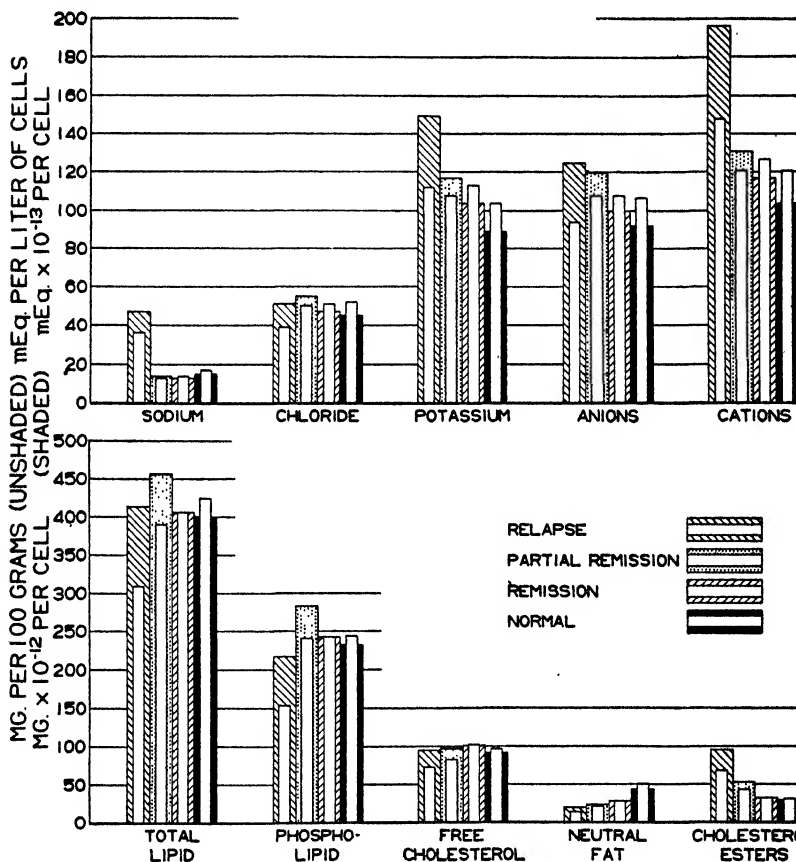
CHART II.   Mineral and lipid distribution of erythrocytes in pernicious anemia.

nevertheless, by calculations made from determinations on whole blood and plasma, demonstrated the appearance of cholesterol esters in the red blood cells of dogs with experimental anemia where normally they were absent.

In the present study significant abnormalities are noted in the erythrocytes during relapse (Table II) which are demonstrated by a high concentration of cholesterol esters and a decreased amount of phospholipid and free cholesterol. With remission the concentration of these lipids returns to normal. The changes from relapse to remission on the basis of average values in mg. per 100 gm. of cells are as follows: 153 to 243 for phospholipid, 14 to 29 for neutral fat, 73 to 102 for free cholesterol, and 69 to 33 for cholesterol ester. In normal erythrocytes approximately 15 per cent of the total cholesterol is in the form of esters, while in the cells of pernicious anemia there is 36 per cent in the ester fraction. After improvement of the anemia with therapy the percentage of ester in total cholesterol becomes normal. Similarly there appear to be abnormalities in the phospholipid make-up of the pernicious anemia erythrocyte. In relapse 70 per cent of the phospholipid is soluble in absolute alcohol, while in remission 81 per cent is soluble, a value similar to that found in the erythrocytes of normal blood (9).

The advantage of expressing the erythrocyte concentration in terms of an individual corpuscle rather than on the unit weight basis has been demonstrated in the anemias (10). This is particularly evident in pernicious anemia where there are not as many cells per unit weight, owing to the enlarged erythrocyte. The lipid concentrations per 100 gm. of cells and per single cell are shown graphically in Chart II by superimposed columns, the heights of which represent the average values (Table II). On the unit weight basis the erythrocytes have less total lipid than those in remission or normal while this difference is not apparent in terms of concentration per single cell. The erythrocyte appears to have a more normal quota of phospholipid and free cholesterol on the basis of corpuscular composition in contrast to indications of lowered amounts in terms of unit weight. Outstanding are the cholesterol esters which are abnormally high in relapse calculated on either basis, while in remission they fall to normal levels. These observations emphasize the view that in pernicious anemia, at least, results of analyses of the erythrocytes on the unit weight basis rather than on the concentration per cell may lead to erroneous conclusions, a finding which will be referred to in the discussion of minerals.

Irrespective of calculating analyses of erythrocytes per 100 gm. or per corpuscle, the percentage composition of the total lipid in terms of its constituent lipid fractions is the same for both and is therefore a measure of derangement in the lipid mixture. There is a low percentage of total lipid as phospholipid and a high percentage as cholesterol esters in relapse (Chart I). After therapy there is a return to normal in both lipid fractions, the phospholipids increasing and the cholesterol esters decreasing. The changes in the free cholesterol and neutral fat are small, although the neutral fat fraction does show a consistent increase towards normality with remission.

Minerals[7]—The distribution of sodium, potassium, and chloride in the serum and erythrocytes (milli-equivalents per liter), together with calculations of the corpuscular composition, is presented in Table III. All observations on the electrolyte composition of the serum are within a normal range of variation whether in relapse or remission. These results are in agreement with those of Cameron (40) on plasma chloride, and those of Emerson and Helmer (41) which demonstrated no derangements of plasma acid-base equilibrium in pernicious anemia.

On the other hand, abnormalities occur in the mineral composition of the erythrocytes, as shown in Table III and Chart II, particularly when the values are converted to concentrations per single red blood cell. As pointed out in the discussion on lipids, the erythrocytes are characteristically large in relapse, and therefore a liter of cells includes a much smaller number than in remission when the erythrocyte size is approximately normal; in some instances only one-half the number of cells is represented in relapse as compared to remission. Consequently this discussion is confined to the corpuscular composition ($\times$ 10^{-13} milli-equivalent per cell) which brings out differences masked in the unit volume concentrations.

Sodium and potassium are elevated in the cells during relapse, as shown by an average of 47 and 149 $\times$ 10^{-13} milli-equivalent per

[7] The normal values represent an average from values reported in the literature by Kramer and Tisdall (35), Oberst (36), Butler and MacKay (37), Hoffman and Jacobs (38), Peters (12), Maizels (39), and Erickson, Williams, Hummel, and Macy (9).

TABLE III

Distribution of Sodium, Potassium, and Chloride of Blood in Pernicious Anemia before and after Therapy

Date	Subject*	R.b.c.	Serum			Erythrocytes			Corpuscles					
			Sodium	Potassium	Chloride	Sodium	Potassium	Chloride	Sodium	Potassium	Chloride	Total cations	Total anions‡	Cations minus anions
		millions per c.mm.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	$\times 10^{-13}$ m.-eq. per cell	$\times 10^{-13}$ m.-eq. per cell	$\times 10^{-13}$ m.-eq. per cell	$\times 10^{-13}$ m.-eq. per cell	$\times 10^{-13}$ m.-eq. per cell	$\times 10^{-13}$ m.-eq. per cell
Apr. 25, 1935	Md§	0.78	133	3.3	104	42	145	27	62	213	40	275	146	129
Feb. 8, 1936	Mb	1.58	137	4.3	101	89	124	44	98	137	48	235	132	103
May 8, 1936		4.60	142	3.8	101	2	120	48	2	124	50	126	102	24
Dec. 10, 1935	Lg	1.58	148	4.6	91	27	118	45	47	204	78	251	166	85
" 23, 1935		3.51	150	5.1	105	21	115	45	22	118	46	140	111	29
Feb. 20, 1936		4.49	139	4.6	94	25	107	43	25	104	42	129	94	35
May 15, 1936		5.25	138	4.6	105	20	121	52	17	103	44	120	89	31
June 3, 1935	Sh	1.69	134	4.0	102	8	87	49	9	96	54	105	112	−7
July 26, 1935		3.88			100			24			24			
May 11, 1936	On	4.47	136	3.8	98	14	112	57	13	106	54	119	126	−7
Jan. 3, 1936		1.80	145	4.3	106	20	122	58	30	183	87	213	173	40
" 15, 1936		3.38	151	4.3	98	0	115	57	0	127	64	127	122	5
May 13, 1936		3.54	141	4.3	104	9	119	58	10	129	63	139	120	19
Feb. 21, 1936	Hn	4.14	138	4.1	97	26	112	50	27	119	54	146	110	36
July 28, 1936		3.49	136	3.8	97	6	81	45	7	99	55	106	118	−7
May 5, 1936		3.63	137	3.3	85	13	107	70	14	117	76	131	135	−4
Jan. 24, 1935	Sr	2.20	117	4.3	93	33	111	0	38	126	0	164	42	122
Apr. 4, 1935		5.90	141	2.1	107	9	105	55	7	85	44	92	87	5

Averages													
Relapse (r.b.c. less than 2.5 millions per c.mm.)	1.68	136	4.3	99	36	112	39	47	149	51	196	125	71
Partial remission (r.b.c. 2.5 to 4.5)	3.65	142	4.2	98	13	108	50	14	117	55	131	120	11
Remission (r.b.c. more than 4.5)	4.94	139	3.8	101	14	113	51	13	104	47	117	100	17
Normal‖		140	4.4	103	17	104	52	15	89	45	104	92	12

* The sequence of date, age, and sex is the same as in Table I.
† Represents the concentration in an average single red blood cell.
‡ See foot-note 8.
§ See foot-note 2.
‖ See foot-note 7.

cell, respectively, as compared to the normal values of 15 and 89×10^{-13} milli-equivalent. During the partial remission stage the sodium content of the cell is normal, although the potassium is still elevated at 117×10^{-13} milli-equivalent, but is only slightly higher than normal in remission with 104×10^{-13} milli-equivalent per cell. The potassium content of the erythrocyte parallels the corpuscular hemoglobin concentrations, a relationship which has been noted by Hoffman and Jacobs (38). The chloride content of the erythrocyte during relapse and partial remission is slightly higher than in remission or normal.

The abnormalities in the total cation, *i.e.* the sum of sodium and potassium content of the cells, is likewise shown in Chart II. The total base is increased in relapse but diminishes under treatment with the attainment of approximate normality in remission. A decrease of base and an increase of water in erythrocytes following treatment of pernicious anemia has been noted by Henderson (42) and Maizels (39), yet the initial level of base in relapse was reported to be low or normal. Approximate calculations[8] of the anion content of the erythrocytes, from the corpuscular hemoglobin, chloride, and bicarbonate, also demonstrate elevated values in relapse, decreasing amounts with treatment, and approximately normal concentrations in remission (Table III and Chart II). These observations indicate that in relapse the cell cation is increased out of proportion to the elevation of cell anion, thus forming a marked excess of cations, approximating 71×10^{-13} milli-equivalent per cell, as compared to normal values (12×10^{-13} milli-equivalent per cell) in partial and complete remission. Maizels (39), observing an excess of cation in the erythrocytes of microcytic and macrocytic anemias, has suggested the presence of greater amounts of an important undetermined anion X^- in anemia which is reduced to normal after treatment. An augmentation of excess cations in the erythrocyte has been observed in other types of anemia (10), particularly in erythroblastic anemia.

[8] The normal concentration of bicarbonate in the cells (12) was used for all calculations; the combining equivalents of hemoglobin were calculated from the corpuscular hemoglobin on the basis of 45 equivalents for hemoglobin per liter of normal cells (12).

Comment

In a previous paper (10) the relationship of lipid and mineral composition to the physiological activity and structure of the erythrocyte in the anemias of childhood has been discussed. The pathological red blood cell of pernicious anemia, in which there is a return to normal chemical composition accompanied by correction of hematological abnormalities during the course of specific therapy, offers an additional opportunity of studying these relationships.

Bloor and his school (43–45) have demonstrated that greater physiological activity in a tissue or organ is associated with increased amounts of phospholipid and free cholesterol, and on the contrary lowered physiological activity, degeneration, and retrogression are accompanied by decreased quantities of these particular lipids and augmented amounts of neutral fat and cholesterol esters. In this connection Gérard (46) has suggested that higher potassium-sodium ratios in tissues are related to a higher state of activity.

In the present study, outstanding was the finding of a large amount of cholesterol ester in the erythrocytes of pernicious anemia which diminished under treatment and became normal with remission. The phospholipid content which was generally lower in relapse increased with treatment. The corpuscular phospholipid is elevated even above normal in the stimulated stage of recovery (partial remission) which is characterized by an influx of young cells. Free cholesterol also was found to be consistently higher in remission. Calculation of the potassium-sodium ratios of the cells gave values of 3.1, 8.3, and 8.1 for relapse, partial remission, and remission respectively as compared to a ratio of 6.1 for the normal erythrocyte.

If one accepts the current views with regard to physiological activity, the lipid and mineral composition of the abnormal erythrocyte in pernicious anemia relapse indicates that the cells are not only in a state of lowered function or activity, but degenerating and retrogressing.

Although it has been pointed out that physiological activity is not confined to oxidative reactions alone but to all the processes of

cellular function (43), it is significant that respiration studies of the erythrocytes in severe relapse indicate diminished oxidative or fermentative metabolism (47). The stimulative effect of "respiratory supplements" on cell respiration rates has been found to be lower than normal, an abnormality which has been attributed to alterations in the structure of the red blood cell membrane (48). Henderson (42) has shown that the hemoglobin of pernicious anemia cells has a smaller affinity for oxygen than hemoglobin of normal blood, thus indicating an inefficiency in the respiratory function of the red blood cell and its hemoglobin. These comments are in accord with the clinical picture of the disease and the changes in chemical composition effected by therapy.

SUMMARY

Coordinated hematological, chemical, and physicochemical observations have been made on the blood of patients suffering from pernicious anemia and during the course of recovery of these subjects after therapy.

The chemical studies have included the complete lipid distribution together with the sodium, potassium, and chloride content of the serum and erythrocytes. The chemical composition of the cells has been calculated in terms of the mean concentration per single red corpuscle, which computation brings out the importance of such a procedure in interpreting the results of chemical analyses of the erythrocytes in the blood dyscrasias.

The serum minerals appear to be unaffected in pernicious anemia, but the plasma lipids are characterized by an increased amount of neutral fat with a concomitant deficiency of cholesterol esters and phospholipid, which return to normal levels after therapy.

The hematological and physicochemical studies indicate that the macrocyte of pernicious anemia is slightly more spherical and that the degree of spherocity, the cell volume, and corpuscular hemoglobin are related. Contrary to general belief, the chemical composition of the erythrocytes exhibits striking abnormalities which are indicative of a deficient corpuscular structure. The red cells of pernicious anemia in relapse are characterized by the presence of excessive amounts of cholesterol esters and a defi-

ciency, particularly in terms of percentage composition, of phospholipid and free cholesterol.   Both the cation and anion content are elevated, the former due chiefly to the increased potassium and the latter to a greater hemoglobin content.   After therapy, the chemical composition of the erythrocyte becomes normal concomitantly with the subsequent improvement of the hematological and clinical picture with remission.

The relation of the physiological activity or function of the corpuscle to its chemical composition in pernicious anemia has been discussed in the light of the recovery in the clinical picture after therapy.

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AN IMPROVED METHOD OF PREPARING HEXOSE-MONOPHOSPHATE FROM YEAST EXTRACT*

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The fundamental equation of fermentation in yeast extracts is usually considered to be the Harden-Young equation (2) which states

$$(1) \quad 2C_6H_{12}O_6 + 2PO_4HM_2 = 2C_2H_6O + 2CO_2 + 2H_2O + C_6H_{10}O_4(PO_4M_2)_2$$

According to this equation there is no hexosemonophosphate present, but, as Harden and Young realized, the essential part of this equation is the equivalence between the phosphate esterified and the CO_2 produced. Not all of this esterified phosphate is present as hexosediphosphate, as the equation would show, but a small part is present as hexosemonophosphate. This small amount of monophosphate is the source from which most preparations have been made.

In a recent paper (3) it was reported that the presence of any one of three dyes—naphtholsulfonate indophenol, rosinduline GG, or brilliant alizarin blue—inhibits fermentation of glucose by yeast extracts under aerobic conditions, apparently by repressing the formation of hexosediphosphate. If hexosediphosphate is added to the extracts containing glucose and the dye fermentation returns until the diphosphate added is fermented. However, instead of the inorganic phosphate in the solution increasing, as would be expected from the straight fermentation of hexosediphosphate, it decreases. The following equation is approximately fulfilled.
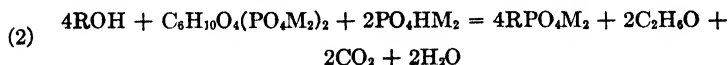
$$(2) \quad 4ROH + C_6H_{10}O_4(PO_4M_2)_2 + 2PO_4HM_2 = 4RPO_4M_2 + 2C_2H_6O + 2CO_2 + 2H_2O$$

Thus the essential feature of the Harden-Young equation, the equivalence between the CO_2 formed and the inorganic phosphate esterified, is maintained, but no hexosediphosphate is formed.

The purposes of the present paper are: to show that the organic phosphate ester formed under the influence of these dyes, the RPO_4M_2 in Equation 2, is chiefly hexosemonophosphate; to show that utilization of this fact affords a considerably improved method of preparing this substance; and to show that this substance is a mixture of glucose-, fructose-, and mannosemonophosphates.

<center>EXPERIMENTAL</center>

The preparation is carried out essentially according to the directions of Warburg and Christian (4), which in turn are based on those of Robison and Morgan (5).

The yeast used was a beer yeast kindly supplied to us by Ruppert's brewery. The wet yeast as it arrived from the brewery was wrapped in burlap and pressed with a hydraulic press at 800 pounds pressure. The cake was then spread out in thin layers and allowed to dry for several days at 25°. This dry yeast was then ground with a Hobart mill (Class DIA-130) to a fine powder and stored in the ice box. Extracts were prepared from this, as required, by treating it with three parts of distilled water at 37° according to von Lebedev (6). The preparation of the monophosphate was carried out at 30°. The extract was placed in a round bottom flask of a capacity several times greater than the volume of extract and gently shaken by a mechanical shaker. The flask was closed by a stopper carrying a dropping funnel and an outlet tube which was immersed in water so that the rate of fermentation could be estimated by the number of bubbles of CO_2 released. 2 gm. of glucose per 100 cc. of extract and a trace of diphosphate were added and the fermentation allowed to start. A solution containing 20 gm. of glucose, 8.8 gm. of anhydrous Na_2HPO_4, and 1.3 gm. of KH_2PO_4 per 100 cc. was then added from the dropping funnel at such a rate that the fermentation was kept at or near the maximum rate. Such a solution is, of course, alkaline, but the esterification of the phosphate changes the pK_2 from 6.8 to a value of 6.1 to 6.2, and this is sufficient to change the pH to the acid side. The optimum amount of this solution

to add has not been tested. We have varied it only from 1 to 1.5 times the volume of yeast extract.

In the preparation without dye, after the solution of glucose and phosphate has been added, the isolation of the product is carried out as Warburg and Christian describe. This consists of the following steps: (1) precipitation of the proteins with trichloroacetic acid, (2) precipitation of the inorganic phosphate and the hexosediphosphate as the barium salts, (3) precipitation of the monophosphate as the lead salt, (4) conversion of the monophosphate to the barium salt, (5) precipitation of impurities as mercury salts, and (6) conversion of the monophosphate to the calcium salt. In place of their *Liquor Plumbi subacetici* we have used Goulard's extract. The decolorization with charcoal was found to be unnecessary. For details their paper should be consulted.

In the dye experiments we have usually used rosinduline GG. This can be obtained commercially from Pfaltz and Bauer, New York. This dye has one distinct advantage over the other two mentioned. This is, that it does not increase the oxygen consumption of the extract. The reaction that occurs is accordingly less complicated. The experiment is just the same as the one without dye until half the solution of glucose and phosphate has been added. Then the remainder of this solution and the fermenting mixture are both made 5×10^{-3} M with dye and the experiment is continued. The rate of addition of glucose is kept the same as before. After it has all been added, the solution is treated just as the one without dye. Part of the dye precipitates with the proteins, part with the first barium precipitate, and part remains in solution. The concentration of monophosphate in the fermenting mixture is increased sufficiently by this procedure so that a part of it may precipitate as the barium salt in the 10 per cent alcohol precipitation. It is necessary, therefore, to extract this precipitate with water. A precipitation of monophosphate in such fractions was noticed by Robison and Morgan (5).

Starting with 250 gm. of dried yeast, which yields about 250 cc. of extract—this quantity was chosen because the amount of material then never exceeds what can easily be handled with ordinary laboratory equipment—the best yield of monophos

phate, as crystalline calcium salt, that I was able to obtain without the use of any dye was 8.0 gm. This yield is equal to only the lower limit of the yield reported by Warburg and Christian. With our yeast as much as 25 per cent of this calcium salt may consist of a non-hexosemonophosphate. This has been identified (7) as glyceromonophosphate. In such a preparation the entire fermentation may be considered to occur according to Equation 1, subject to the limitations stated above.

With the same amount of material, but with the addition of dye, as described above, one can obtain 25 gm. of crystalline calcium salt. In this preparation the diphosphate formed during the first half of the fermentation is converted during the second half into another ester, in accordance with Equation 2. As stated, this increases 3-fold the yield of calcium salt. This calcium salt has a carbon to phosphorus ratio of a little less than 6. It was expected, therefore, that it consisted chiefly of hexosemonophosphate mixed with some glycerophosphate as in the experiments without dye.

It was stated in a previous paper (7) that the conditions for the separation of glyceromonophosphate as a 1:1 mixture with glucosemonophosphate depended on the total ester concentration and on the relative concentration of the glycerol ester. When the latter is less than 10 per cent of the total, as it is in these dye experiments, the first alcohol precipitate, provided this does not make up more than 50 per cent of the total, may contain none of it. It precipitates in the middle fractions, the last one being again free of it. We have followed its presence by the carbon to phosphorus ratio. The carbon was determined by the method of Van Slyke, Page, and Kirk (8) and the phosphorus by the method of Fiske and Subbarow (9). The preparation described previously (7) consisted of an equimolar mixture of glucosemonophosphate and glyceromonophosphate. It is not essential, however, that the hexose in these mixed crystals be glucose. Mixed crystals are also formed with fructose- or mannosemonophosphates. After the glycerol ester has been removed as this 1:1 mixture with hexosemonophosphate, the remainder is our monophosphate preparation. Since the amount of glycerophosphate present never exceeds 10 per cent of the total calcium salt, there are left, after its separation, about 20 gm. of hexosemonophosphate.

This is from 4 to 5 times as much as is obtained without dye after the same kind of fractionation.

In spite of the improved yield, however, the amount of phosphorus isolated as hexosemonophosphate is only a small part of the total phosphorus present. The statement that the reaction in the presence of the dye follows Equation 2 is based, therefore, not on this isolation but on experiments of which the following is an example. In the usual Warburg manometric apparatus, an amount of diphosphate equivalent to 807 c.mm. of CO_2, if completely fermented, was added to a yeast extract containing rosinduline GG and glucose. 856 c.mm. of CO_2 were produced before the fermentation practically ceased. During this fermentation 1.24 mg. of phosphorus disappeared from the inorganic phosphate of the solution. If we express phosphorus in c.mm. as if it were a gas (31 gm. = 22.4 liters), so that it can be compared directly with the CO_2, the 1.24 mg. are equal to 896 c.mm. Thus Equation 2 is approximately fulfilled. The hydrolysis curve of the organic phosphate present indicates that most of it belongs to the difficultly hydrolyzable fraction. It is only 20. per cent hydrolyzed in 1 hour in 1.0 N H_2SO_4 at 100°.

In the large scale experiments hexosemonophosphate can be isolated in amounts equal to 10 per cent of the total phosphorus, although we know that when the experiment is stopped a large part of the phosphorus is present as inorganic phosphate, some as hexosediphosphate, and some as glycerophosphate, and also know that the isolation involves considerable losses. This fact justifies, we believe, the statement that hexosemonophosphate is the chief product formed.

The analysis of this monophosphate after drying *in vacuo* over $CaCl_2$ is as follows:

Pregl,[1] 4.949 mg. sample + $K_2Cr_2O_7$. 3.881 mg. CO_2, C 21.39%; 2.012 mg. H_2O, H 4.55%

Van Slyke, 2.042 mg. sample, PCO_2 312.7 mm., T 25°; C 0.4372 mg., 21.41%

The calcium (de Waard (10)) is 11.78 per cent. The phosphorus (Fiske and Subbarow (9)) is 8.95 per cent.

[1] We are indebted to Dr. Goldforb in the laboratory of Dr. M. Bergmann for this analysis.

	C	H	P	Ca
Found..........................	21.40	4.55	8.95	11.78
Calculated for $C_6H_{11}O_9PCa \cdot 2H_2O$.	21.55	4.49	9.28	11.98

Different preparations and different fractions from one preparation vary only slightly around these values. The water content is, of course, the most variable part. 1 of the 2 water molecules can be removed by prolonged drying over P_2O_5. When 2 water molecules are present, the substance is stable in air. The aldose value (iodometric titration) (11) of different samples varies considerably. It has never been found to be higher than 75 per cent and it may be as low as 56 per cent. The specific rotation is also low in comparison to that of glucosemonophosphate. When determined by dissolving 50 to 60 mg. in 1.0 cc. of 1.0 N HCl, it is around $+20°$ for the D line. The Hagedorn-Jensen value is independent of the aldose value. Determined by using NaOH as the alkali, as suggested by Robison and Morgan (5), it is always 80 per cent that of glucose (*i.e.* equal to that of glucosemonophosphate). When determined with Na_2CO_3 as the alkali, it is a few per cent less. The preparations contain some relatively easily hydrolyzable phosphate; *e.g.*, a preparation with an aldose value of 60 per cent is 17.5 per cent hydrolyzed in 3 hours with 1.0 N H_2SO_4 at 100°. The Seliwanoff reaction, carried out as described by Roe (12), is positive. For example, the preparation just mentioned shows 22 per cent ketose. This value is in agreement with the supposition that the easily hydrolyzable phosphorus is present as fructosemonophosphate.

The above data indicate that the preparation contains glucose- and fructosemonophosphates, but the properties cannot be explained on the basis of these two alone. At least one other substance must be present. This substance must have the analysis of a hexosemonophosphate. It must have the same reducing power toward ferricyanide in the presence of NaOH that glucose- and fructosemonophosphates have, but it must have a lower aldose value and a lower specific rotation than glucosemonophosphate. These are precisely the properties which Robison (13) and Jephcott and Robison (14) ascribe to mannosemonophosphate. They report that it titrates only about 60 per cent as aldose and has a much lower rotation ($[\alpha]_{546}^{\text{free acid}} = +15.1°$). They also show that mannosemonophosphate forms an insoluble

phenylhydrazine salt of the phenylhydrazone and may be obtained in this way from a mixture with the glucose- and fructose-monophosphates. Our preparations readily form an almost insoluble hydrazone at room temperature. After being recrystallized twice from 75 per cent alcohol, it melts at 144° with a preliminary darkening beginning at about 135°. The melting point reported (14) is 144–144.5°. Our preparation, therefore, contains mannosemonophosphate. However, we obtained this hydrazone in a yield of only 7.0 per cent of the phosphorus present. If we assume that the amount present was far greater than this yield, then the properties of our preparations can be explained on the basis of a mixture of glucose-, fructose-, and mannosemonophosphates. Thus the preparation with an aldose value of 60 per cent and a ketose value of only 22 per cent would have to contain glucose and mannose in about equal parts. This would also account for the $[\alpha]_D^{free\ acid} = +20.2°$.

SUMMARY

An improved method of preparing hexosemonophosphate from yeast extracts is described. This preparation is shown to be a mixture of glucose-, fructose-, and mannosemonophosphates.

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THE EFFECT OF CYSTEINE ON HEREDITARY HYPOTRICHOSIS IN THE RAT (MUS NORVEGICUS)

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In a very interesting paper Martin and Gardner (1) reported that the administration of cystine stimulated hair growth in the hypotrichotic rat and cysteine produced a complete coat of hair within 2 weeks, which was maintained for 1 month, at which time the experiment was discontinued.

·In 1931 some of our hypotrichotic rats were fed cystine in our laboratory of animal nutrition[1] with negative results.

Since the publication of Martin and Gardner's work, twenty-four hypotrichotic rats have been fed cysteine hydrochloride, the results of which are given in Table I. These animals were paired with litter mates of the same sex for controls. Fourteen of the twenty-four pairs were males and ten were females. Among our stock animals a great deal of variation in hair growth has been observed, the ability to grow hair decreasing with age. Also the amount of hair varies with families. This makes it desirable to have, as controls, animals of the same age, sex, and of as nearly the same breeding as possible.

The stock ration, essentially the same as used by Martin and Gardner, contained the following ingredients.

	gm.		*gm.*
Wheat (soft)	908	Whole milk powder	1135
Corn (yellow)	908	Calcium carbonate	23
Oats (rolled)	908	Sodium chloride	45
Flaxseed oil meal	454	Iron citrate	5
Casein (crude)	159	Copper sulfate	2

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[1] This work was done by Dr. D. B. Smuts, Onderstepoort, Pretoria, South Africa.

In some of the tests the cysteine was dissolved in water and dropped on the stock diet. In the others dry cysteine hydrochloride was fed with the ration. In all cases the cysteine was consumed in a few hours.

The cysteine hydrochloride was prepared by the Division of Organic Chemistry, University of Illinois. It was shown by tests that oxidation of the cysteine hydrochloride before consumption did not occur.

TABLE I

Comparative Analyses on Hypotrichotic Rats Fed Cysteine Hydrochloride
There was no increase in the amount of hair.

No. of pairs	Initial age	No. of days fed	Cysteine hydrochloride fed daily to one member of each pair
	days		*mg.*
5	48	35	5*
3	42	35	5*
2	37	35	5*
5	31	21	6.5†
3	30	21	6.5†
2	30	21	6.5‡
2	28	21	6.5‡
2	30	17	6 5‡

* Dissolved in distilled water. After 18 days fed 6.5 mg. for 17 days.
† Dissolved in distilled water first 7 days. Fed in dry form 14 days.
‡ Dissolved in distilled water.

DISCUSSION

I have no explanation for the negative results obtained in this experiment when positive results were found by Martin and Gardner. The rats used by Martin and Gardner came from stock sent to the Johns Hopkins laboratory from our laboratory. Since that time our stock has been crossed with the hypotrichotic mutant strain originating at the Ohio Experiment Station, described by Wilder, Bethke, Kick, and Spencer (2). These two strains, however, are the same genetically (Roberts (3, 4) and Feldman (5)). Unpublished breeding tests in our laboratory also confirm this.

Martin and Gardner interpret their results as an indication of a hereditary absence in the hypotrichotic rat "of an enzyme capa

ble of breaking glutathione or other peptide linkages involving
sulfur-containing groups into the constituent amino acids."

Skin from a normal rat transplanted to a hypotrichotic one
will grow and maintain normal hair (Fig. 1).

If the conclusion of Martin and Gardner is correct, it follows
that cells of the skin possess the function of breaking these com-
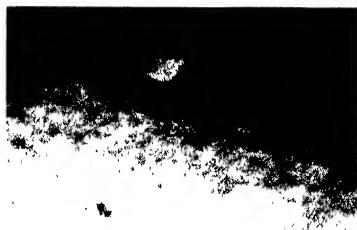
Fig. 1. Showing normal hair produced on skin from a normal rat trans-
planted to a "hairless rat."
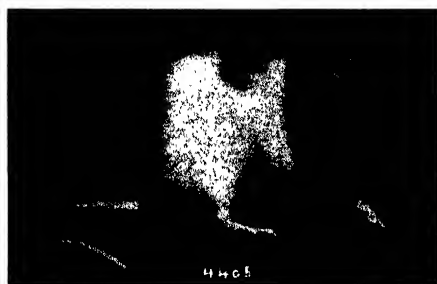
Fig. 2. Female 4405 was hairless at 5 weeks of age but later regained a
normal growth which was retained throughout life. Discarded at the age
of 621 days. Its genetic constitution was Hh.

pounds into the constituent amino acids; otherwise the normal
skin transplant on the hypotrichotic rat would not maintain
normal hair, if cysteine is necessary for hair growth. The result
of the transplant indicates that the cause of the hypotrichotic
condition in the rat, whatever it may be, lies in the skin itself.

One animal in our laboratory, which in its early life was as hair-

less as any we have had, later grew a normal coat of hair and maintained it for several months until death (Fig. 2). In testing the genetic constitution of this animal by mating with a "hairless" (hh) one it was found to be heterozygous (Hh), producing both Hh and hh offspring. This suggests the possibility of a genetic level or threshold below which an Hh individual may not have a normal coat of hair. This is the only case among hundreds of animals which we have had behaving in this manner, and therefore would be an improbable explanation of the differences in our results.

SUMMARY

From these controlled feeding experiments no evidence was obtained that cysteine hydrochloride added to a complete stock diet affected growth of hair in the hypotrichotic rat (*Mus norvegicus*).

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THE SULFUR DISTRIBUTION AND BASIC AMINO ACIDS OF LIMULUS HEMOCYANIN

By ABRAHAM MAZUR

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Little is known of the forms in which sulfur is combined in the hemocyanin of *Limulus polyphemus*. The figures for nitrogen distribution secured by Van Slyke (1) indicate the presence of 1.10 per cent of cystine in the protein; this corresponds to less than 27 per cent of the total sulfur on the basis of the value (1.10 per cent) determined by Hernler and Philippi (2), and to an even smaller proportion of that (1.56 per cent) reported by Alsberg and Clark (3).

The experiments here reported indicate that of the 1.22 per cent of sulfur in the sample of hemocyanin examined, 0.52 or 47.5 per cent is in the form of cystine and 0.60 or 49 per cent is in the form of methionine; the remaining 3.5 per cent is converted into hydrogen sulfide on reduction by hypophosphite in hydriodic acid. According to Conant, Dersch, and Mydans (4) the black pigment produced by the action of alkali upon hemocyanin contains neither methionine nor cystine, but gives up all its sulfur, in the elementary form and as copper sulfide, on boiling with acid; this fraction may conceivably be the same as that responsible for the hydrogen sulfide.

The total nitrogen content of hemocyanin is found to be 17.5 per cent, in good agreement with the figure (17.3 per cent) reported by Redfield, Coolidge, and Shotts (5), and higher than the values found by other workers: 16.9 (Hernler and Philippi), 16.18 (Alsberg and Clark). Determination of the basic amino acids by the micromethod of Block yields values (Table I) for arginine and lysine in fair agreement with those reported by Van Slyke (1) and by Roche and Jean (6); that for histidine, however, is only about one-half of those previously recorded.

The *Limulus* hemocyanin was a highly purified product kindly furnished by Professor A. C. Redfield in the form of a 10 per cent aqueous solution. The protein was precipitated with 5 volumes of absolute alcohol, washed with ether, and dried over phosphorus pentoxide *in vacuo*. Analyses showed 17.5, 17.5 per cent (Kjeldahl) of nitrogen, and 1.22, 1.21, 1.22 per cent (Pregl), 1.23 per cent (Zahnd-Clarke) of sulfur.

TABLE I

Percentage Basic Amino Acids from Limulus Hemocyanin

	Van Slyke[*]	Roche and Jean	Present report
Arginine..........................	7.84	6.85	6.37
Histidine.........................	7.83	9.08	4.52
Lysine............................	7.09		8.92

[*] The figures attributed to Van Slyke have been calculated on the assumption that his hemocyanin contained 16.04 per cent of nitrogen, the value indicated by his own data.

Cystine[1]

A sample of the dried protein weighing 0.7852 gm. was heated with 10 cc. of 20 per cent hydrochloric acid at 135° in an oil bath for 8 hours in an all-glass reflux apparatus. The hydrolysate and washings were diluted to 25 cc. and filtered. Aliquots of this solution were used for the Sullivan and Folin-Marenzi determinations.

Sullivan Method—2 cc. aliquots of the above hydrolysate were treated according to the modification of the Sullivan method by Brand, Harris, and Biloon (7), use being made of the photoelectric colorimeter. Values were obtained of 1.83 and 1.86 per cent, average 1.85 per cent, of cystine, corresponding to 0.49 per cent of cystine sulfur.

Folin-Marenzi Method—1 cc. aliquots of the same hydrolysate were treated according to the method of Folin and Marenzi as modified by Kassell (8), the Pulfrich photometer being employed. No cysteine was detected. Both determinations gave values of

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[1] The author wishes to express his thanks for the aid given by Miss Beatrice Kassell and Dr. E. Brand in the determinations performed by their modifications of the methods of Folin, Sullivan, and Baernstein.

1.94 per cent cystine, corresponding to 0.52 per cent of cystine sulfur.

Methionine and Cystine

Baernstein Method (9)—The hemocyanin was extracted thoroughly with light petroleum and dried *in vacuo* over phosphorus pentoxide at 100° to remove any trace of alcohol or ether, which would yield volatile iodide. Samples (0.257 and 0.260 gm.) of this product were treated with 10 cc. of 57 per cent hydriodic acid and the cystine was determined as cysteine by tetrathionate titration. Corrected values of 1.96 and 1.91 per cent were obtained, average 1.94 per cent, of cystine or 0.52 per cent cystine sulfur. Determination of the volatile iodide in the duplicate determination gave identical corrected values of 2.76 per cent methionine or 0.60 per cent methionine sulfur. Determination of the methionine as homocysteine (formed by the action of the hydriodic acid) gave corrected values of 2.54 and 2.52 per cent; average 2.53 per cent methionine or 0.54 per cent methionine sulfur. This value is known to be less reliable than that obtained from the volatile iodide, but serves as a check. The "sulfate S," determined in the Baernstein method as hydrogen sulfide, gave duplicate corrected values of 0.08 per cent.

Alkali-labile sulfur was determined on the dry protein according to the procedure of Blumenthal and Clarke (10). Values of 0.50 and 0.52 per cent, average 0.51 per cent, were obtained. This figure corresponds well with those for cystine sulfur.

Nitric acid-oxidizable sulfur was determined by the method of Blumenthal and Clarke (10). Duplicate determinations gave values of 0.54 and 0.52 per cent, average 0.53 per cent. This value also checks with the cystine sulfur figures.

No sulfate was produced by the action of bromine (10).

The total sulfur accounted for as methionine (0.60 per cent), cystine (0.52 per cent), and "sulfate S" (0.08 per cent) equals 1.20 per cent. This corresponds very well with the total sulfur (Pregl) of hemocyanin (1.22 per cent).

Basic Amino Acids

These were determined according to the method of Block (11), 2.50 gm. portions of protein being hydrolyzed with 25 cc. of 1:3.5 sulfuric acid for 24 hours.

Histidine—Yield, 566.7 mg. and 574.2 mg. of histidine diflavi-anate, corresponding to 4.49 and 4.55 per cent of histidine respectively; average 4.52 per cent.

Arginine—Yield, 452.8 mg. and 439.4 mg. of arginine mono-flavianate, corresponding to 6.46 and 6.27 per cent of arginine; average 6.37 per cent.

Lysine—Yield, 577.2 mg. and 565.9 mg. of lysine picrate, corresponding to 9.00 and 8.83 per cent of lysine; average 8.92 per cent.

The author wishes to express his thanks to Professor H. T. Clarke for suggesting this problem, and for his encouragement and advice.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM

XI. MAN AT REST

By D. B. DILL, H. T. EDWARDS, and W. V. CONSOLAZIO

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Our knowledge of the physicochemical properties of human blood has been extended in several directions in recent years. A tabular description of oxygenated blood has been given by Henderson, Dill, Edwards, and Morgan (1) (Paper X of this series). We are indebted to Roughton (2) for much new information about carbamino-CO_2 ($HbCO_2$); estimates of its concentration in oxygenated and reduced cell hemolysate have been made by Ferguson and Roughton (3) and by Ferguson (4). The revision by Dill, Daly, and Forbes (5) of the pK′ values for oxygenated and reduced cells makes possible a recalculation of the cell pH and of its change in the respiratory cycle. These advances, together with new data dealing with the distribution of electrolytes between serum and cells, have been used in this paper to synthesize a description of normal human blood by the method of Henderson (6).

It is planned to use this study as a basis of reference for determining the effects of high altitude on the physicochemical properties of the blood. Accordingly, we have used as subjects, in so far as they were available, the members of the International High Altitude Expedition of 1935 either before or several months after their stay in high altitudes.

The methods used for equilibration of blood and its analysis in the Van Slyke apparatus have been described before (6). The oxygen-combining power of blood is determined on the Van Slyke apparatus after equilibrating the blood with air for 20 minutes at 20°. Dissolved oxygen is estimated from the solubility factor established by Sendroy, Dillon, and Van Slyke (7). Water content of serum and cells is determined on weighed samples by

drying to constant weight at 110°.   Serum protein is determined by micro-Kjeldahl analysis.   Other methods used are for chloride, that of Van Slyke (8); for sodium, Butler and Tuthill (9); for potassium, Shohl and Bennett (10); for calcium, Kramer and Tisdall (modified by Clark and Collip (11)); and for lactic acid, Friedemann, Cotonio, and Shaffer (12).

Venous blood for studying the distribution of anions between serum and red cells was obtained from fourteen men of whom
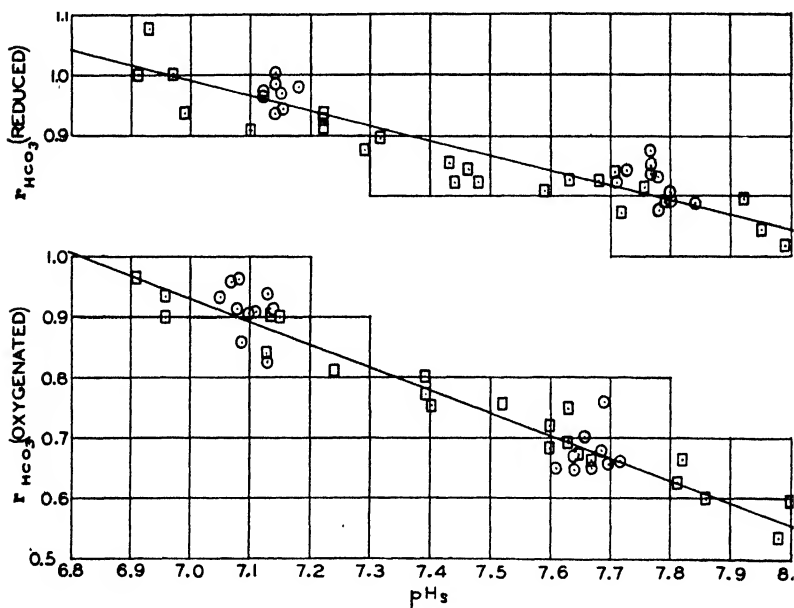
FIG. 1. The distribution of combined CO_2 between cells and serum of human blood in relation to pH and degree of oxygenation.   The squares correspond to experiments of 1930–31; the circles, 1935–36.

seven made the Chilean trip.   The distribution of combined CO_2[1] in relation to pH_s and oxygenation, determined in samples

[1] $r_{HCO_2} = \dfrac{(BHCO_3)_c}{(BHCO_3)_s} \times \dfrac{(H_2O)_s}{(H_2O)_c}$ where $(BHCO_3)$ represents the difference between total CO_2 and free CO_2; it includes all forms of combined CO_2. Concentrations of electrolytes are expressed in this paper in milli-equivalents.   The subscripts c, s, and b refer to cells, serum, and blood, respectively.   Concentration of water is in gm. per liter of serum or of cells.

equilibrated with suitable mixtures of CO_2 and O_2, is shown in Fig. 1. The straight lines have been fitted in accordance with the usual regression equation. The curve for oxygenated blood fits the values given in Paper X very closely. The value for r_{HCO_3} at pH_s 7.38 is 0.790 according to our data, 0.799 according to Paper X, and 0.786 according to Hastings, Sendroy, McIntosh, and Van Slyke (13). While no such comparison is possible in the case of reduced blood, the deviation of points from the line
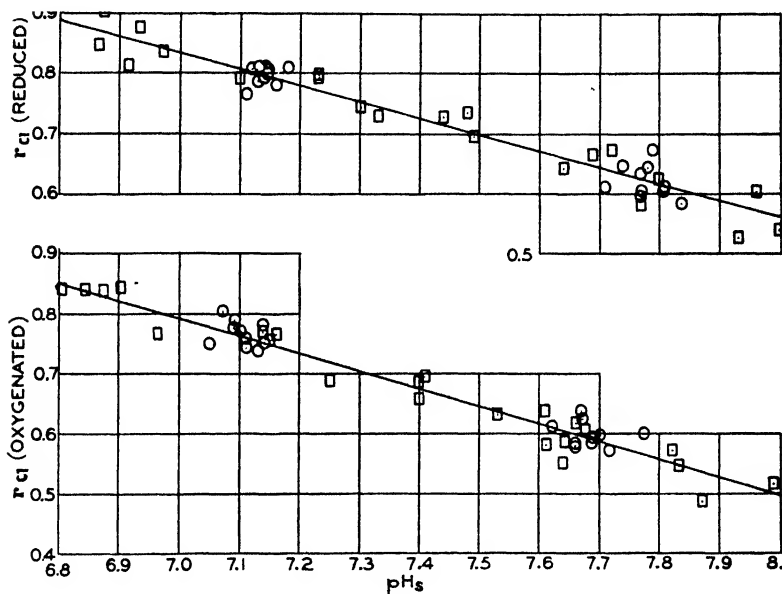
FIG. 2. The distribution of chloride between cells and serum. The squares correspond to experiments of 1930–31; the circles, 1935–36.

is of the same order of magnitude as in oxygenated blood. The notable feature of the pair of curves is their wide divergence in the alkaline range, a characteristic not in accord with the theory proposed by Van Slyke, Wu, and McLean (14). The significance of $HbCO_2$ in this connection will be discussed below.

The distribution ratios for the chloride ion are shown in Fig. 2. The curves, derived from the data in the same manner as those of Fig. 1, are in better accord with the theory of Van Slyke, Wu,. and McLean (14) than are the corresponding curves for combined

CO_2.   According to their theory, r for anions has a value of 0.73 in oxygenated blood at a pH_s of 7.38.   Our corresponding value for r_{Cl} is 0.682 and that of Hastings, Sendroy, McIntosh, and Van Slyke (13) is 0.689.   The points for r_{Cl} in reduced blood show no great deviation from the line; the precision of the analytical methods used seems to be independent of the degree of oxygenation.

The pH of red cells, pH_c, has been calculated by use of the Henderson-Hasselbalch equation with the values for pK'_c given
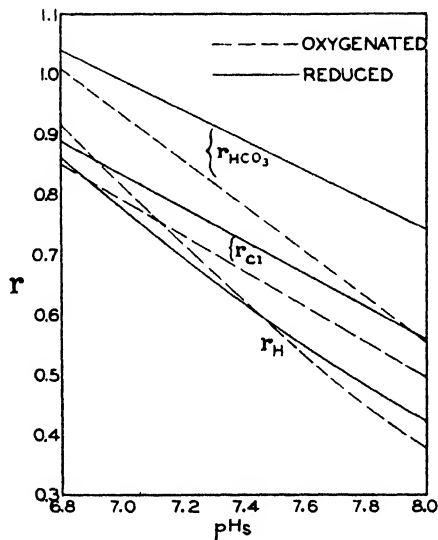
Fig. 3. Smoothed curves representing the distribution of combined CO_2, chloride, and the hydrogen ion.

by Dill, Daly, and Forbes (5).   The distribution of the hydrogen ion in relation to pH_s has then been calculated.   The smooth curves of Fig. 3 show the results of the calculation.   Comparison may be made with corresponding curves for r_{HCO_3} and r_{Cl}.   We see that r_H is unique in that the value for reduced blood is less than that for oxygenated blood in the alkaline range.

The foregoing experiments provide a basis for calculating the effect of oxygenation on the base-binding capacity of hemoglobin.   In one series of experiments CO_2 dissociation curves were derived for normal blood and blood with more or less than the

usual proportion of hemoglobin. In each of six such experiments $(BHCO_3)_b$ was plotted as a function of pH_s in the oxygenated and reduced states. From the curves drawn through these points values were derived for $\Delta(BHCO_3)_b$, the increment in base bound due to oxygenation of hemoglobin, at pH_s values of 6.8, 6.85, 6.9 . . . 8.0. After the results were tabulated, the means shown in Fig. 4 were obtained. In another series of experiments on normal blood from ten men, of whom seven were in the Chilean party, equilibration was carried out at pH_s values near 7.1 and
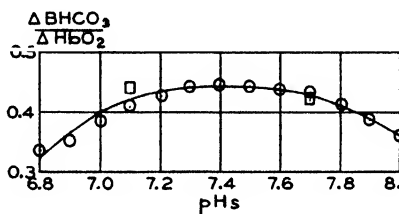
FIG. 4. Effect of pH_s on base-binding capacity of whole blood
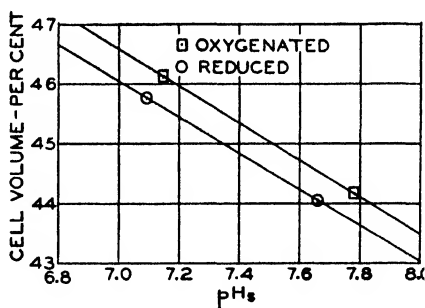
FIG. 5. The relation between pH_s and cell volume in oxygenated and reduced blood.

7.7. The means for $\Delta(BHCO_3)_b$ derived from these experiments are shown in Fig. 4. The weighted curve gives a satisfactory measure of the effect of oxygenation on the base-binding capacity of human blood in the physiological range.

In the set of ten experiments just referred to we have obtained a measure of the effect of pH_s on cell volume in both the reduced and oxygenated states. The curves given in Fig. 5 correspond to the averages of these measurements.

Observations necessary for setting up a standard for resting

blood are contained in Table I.   These specimens of arterial blood represent nine of the ten members of the high altitude party and three other men frequently used as experimental subjects.   A few comparisons with other studies in the literature may be useful. The HbO_2 capacity is slightly less than that reported by Price-Jones (15) for twenty men, with the same method.   The pH and the total CO_2 of blood and serum are close to the means for thirty-nine men reported by Shock and Hastings (16).   The pCO_2 is considerably lower than theirs, but this is in part due to difference in methods; if their data are recalculated to $37°$, a mean pCO_2 of about 42 mm. is obtained.

The relation between total Hb of blood, measured in mM of combined O_2, and V_c, the volume of cells in 1 volume of blood, which may be derived from Table I, will prove useful at a later time.   For arterial blood it is

$$V_c = 0.05 \text{ (total Hb)} \tag{1}$$

The corresponding relation for serum volume is

$$V_s = 1 - 0.05 \text{ (total Hb)} \tag{2}$$

The changes in composition of standard blood with changes in pH_s may be calculated from Table I and Figs. 1 to 5 by using as a beginning the synthetic method developed in Paper X.

The titration curve for a solution of HbO_2 of 9 mM concentration is calculated from Table I, Column 7, of Paper X and tabulated as in that table for pH_s values 6.8, 6.85 . . . 8.0.   The titration curve for plasma is calculated from Column 5 of the same table, the standard value of 39.8 gm. of protein per liter of blood being used.   This value is derived from our Table I by multiplying the concentration of protein in plasma, 72.2 gm. per liter, by the plasma volume, 0.5517.

The two titration curves may then be added, giving a titration curve parallel to that of the specimen of blood in question. This titration curve is then transformed to a CO_2 dissociation curve on the assumption that the base for neutralizing carbonic acid is all supplied by protein; that is, $\Delta BP = -\Delta BHCO_3$.   It is convenient to subtract each value for BP from the value of BP at the most alkaline pH_s, 8.0.   This curve may then be brought to the level of the "standard" blood in question by reference to

TABLE I

Blood of Twelve Men

Subject	Arterial																	Oxygenated at pCO_2 = 40 mm. Hg		
	HbO_2 capacity, mM per l.	HbO_2 content, mM per l.	HbO_2 content, per cent	$(\text{Total CO}_2)_b$, m.-eq.	pCO_2, mm. Hg	$(H_2O)_c$, gm. per cc.	$(H_2O)_g$, gm. per cc.	$(\text{Protein})_g$, gm. per l.	$(HCO_3)_g$, m.-eq.	$(Cl)_g$, m.-eq	$(\text{Lactate})_g$, m.-eq.	$(Na)_g$, m.-eq.	$(K)_g$, m.-eq.	$(Ca)_g$, m.-eq.	pH	$(BHCO_3)_b$, m.-eq.	V_c, cc. cells per cc. blood	$(\text{Total CO}_2)_g$, m.-eq.	$(\text{Total CO}_2)_g$, m.-eq.	$(\text{Total CO}_2)_g$, m.-eq.
Barron	9.22	8.80	95.4	20.94	44.0	0.717	0.937	73.0	23.9	103.7	1.2	143.5	6.5	5.5	7.35	19.67	0.4450	20.0	24.1	14.9
Christensen	8.63	8.22	95.2	22.10	41.9	0.724	0.938	73.1	25.1	104.0	1.4	141.0	5.0	4.7	7.39	20.89	0.4235	21.6	25.9	15.8
Dill	7.84	7.46	95.1	22.20	42.0	0.732	0.938	72.5	25.1	104.8	1.3	137.4	5.0	4.8	7.39	20.99	0.4092	21.7	25.6	16.0
Edwards	9.16	8.86	96.7	21.43	40.8	0.724	0.937	73.4	24.6	105.7	1.5	143.1	4.9	5.0	7.40	20.25	0.4593	21.1	25.5	15.9
Forbes	9.02	8.74	96.9	22.42	38.0	0.716	0.936	74.4	25.8	105.5	1.2	140.6	4.9	4.9	7.44	21.30	0.4496	22.7	27.5	16.8
Hall	9.80	9.34	95.3	21.31	41.0	0.722	0.941	65.8	25.8	104.7	1.3	131.9	8.2	5.1	7.41	20.12	0.4750	21.0	25.7	15.8
Keys	8.76	8.38	95.7	22.29	41.8	0.722	0.939	72.6	25.3	104.9	1.5	142.6	4.5	4.8	7.39	21.08	0.4432	21.8	26.2	16.2
McFarland	9.32	8.87	95.2	21.70	41.5	0.714	0.937	73.0	24.8	103.9	1.3	138.6	5.0	5.3	7.39	20.50	0.4640	21.3	25.9	16.0
Talbott	9.29	8.88	95.6	21.89	40.5	0.719	0.938	71.6	25.2	105.5	1.4	141.5	5.3	5.0	7.41	20.53	0.4515	21.6	26.3	15.9
Anderson	8.82	8.43	95.6	22.60	39.5	0.717	0.938	73.7	26.0	105.3	1.4	140.1	5.2	5.0	7.43	21.46	0.4561	22.6	27.3	17.0
Consolazio, F.	9.52	9.06	95.1	21.87	40.5	0.720	0.938	73.6	25.4	104.1	1.5	139.3	4.7	4.8	7.41	20.70	0.4675	21.6	26.4	16.1
Consolazio, W.	8.62	8.16	94.7	21.81	39.7	0.718	0.938	70.0	24.8	103.5	1.9	140.3	4.5	5.0	7.41	20.66	0.4357	21.7	26.1	16.0
Mean	9.00	8.60	95.5	21.88	41.0	0.720	0.938	72.2	25.1	104.6	1.4	140.0	5.3	5.0	7.40	20.69	0.4483	21.56	26.04	16.05

our Table I which shows that arterial blood contains 20.69 milli-equivalents of combined CO_2 at a pH_s of 7.40.   Fully oxygenated blood at the same pH_s contains slightly less, 20.51 milli-equivalents.   Accordingly, the synthetic dissociation curve is brought to this level.

The next step consists in calculating the value for combined CO_2 in serum for each pH_s value.   For this purpose values for serum volume, V_s, and cell volume, V_c, are tabulated in accordance with Fig. 5, and values for serum water, $(H_2O)_s$, and cell water, $(H_2O)_c$, calculated from these values and the standards given in Table I.   This is an algebraic calculation based on the facts that (a) $(H_2O)_s$ and $(H_2O)_c$ are known for a given pH_s value, 7.40; (b) $(H_2O)_b$ is a constant which can be calculated from Table I; and (c) changes in V_c are due solely to movement of water between serum and cells.   Values for r_{HCO_3} in oxygenated blood are then tabulated from Fig. 1. $(BHCO_3)_s$ is then calculated as follows:

$$\frac{(BHCO_3)_c}{(BHCO_3)_s} \times \frac{(H_2O)_s}{(H_2O)_c} = r \qquad \qquad (3)$$

$$(BHCO_3)_c = r \frac{(H_2O)_c}{(H_2O)_s} (BHCO_3)_s = f_1 (BHCO_3)_s \qquad (4)$$

$$(BHCO_3)_c V_c + (BHCO_3)_s V_s = (BHCO_3)_b \qquad (5)$$

$$(BHCO_3)_c = \frac{(BHCO_3)_b}{V_c} - \frac{(BHCO_3)_s V_s}{V_c}$$

$$= C - f_2 (BHCO_3)_s \qquad (6)$$

From Equations (4) and (6) we obtain

$$f_1 (BHCO_3)_s + f_2 (BHCO_3)_s = C$$

$$(BHCO_3)_s = \frac{C}{f_1 + f_2} \qquad (7)$$

From Equation 7 and the values already tabulated $(BHCO_3)_s$ may be calculated for each value of pH_s.

We have next to calculate $(H_2CO_3)_s$.   This is derived from the values for pH_s and $(BHCO_3)_s$ with the Henderson-Hasselbalch equation in which $pK'_s = 6.11$.   Values for pCO_2 are obtained

from the relation

$$H_2CO_3 = 0.0334 (H_2O)_s pCO_2 \qquad (8)$$

in which 0.0334 is the solubility factor for CO_2 in serum water at 37°. $(H_2CO_3)_b$ is calculated by use of the following factor which takes into account the solubility of CO_2 in cells and in serum as well as their relative proportions.

Milli-equivalents $(H_2CO_3)_b = [0.0334 (H_2O)_s V_s + 0.0362 (H_2O)_c V_c] pCO_2$ (9)

For standard human blood the factor in brackets is 0.0290.

(Total CO_2)$_s$ and (total CO_2)$_b$ are then obtained by addition, giving the familiar CO_2 dissociation curves of oxygenated whole blood and of the corresponding true plasma.

Using Fig. 4 we next tabulate the values for $\Delta(BHCO_3)_b$ due to reduction of HbO_2 at pH_s values 6.8, 6.85 . . . 8.0. The sum of $(BHCO_3)_b$ of oxygenated blood and of $\Delta(BHCO_3)_b$ at each pH_s gives the concentration of $(BHCO_3)_b$ in reduced blood. Exactly the same procedure is then followed for reduced blood as that used for oxygenated blood. The $BHCO_3$ and free CO_2 of oxygenated and reduced cells may be derived in simple fashion and, from these values and pK'_c, pH_c may be calculated.

This synthetic method, although much more laborious, gives a more representative description of the CO_2 dissociation curves than can be had from the examination of a single specimen of blood. With this description at hand the nomogram shown in Fig. 6 has been constructed according to Henderson's method (6).

The curve on this nomogram representing pO_2 is based on the oxygen dissociation curve of the blood of A.V.B. described by Dill, Edwards, Florkin, and Campbell (17). The pH_c values were altered to conform to the new pK'_c values and the curve is shifted to pass through the oxygen tension of 26 mm. at half saturation at the reaction of arterial blood. This is the mean value reported by Keys, Hall, and Barron (18) for nine members of the Chilean party at sea-level.

The line representing arterial blood in Fig. 6 is drawn to conform to Table I. In drawing the line for venous blood we have employed Grollman's measurements of cardiac output (19) which indicate that the oxygen transport in rest is 5.90 volumes per cent or 2.65 mM per liter. With an R.Q. of 0.82 the total CO_2 transport is 2.17 mM per liter.
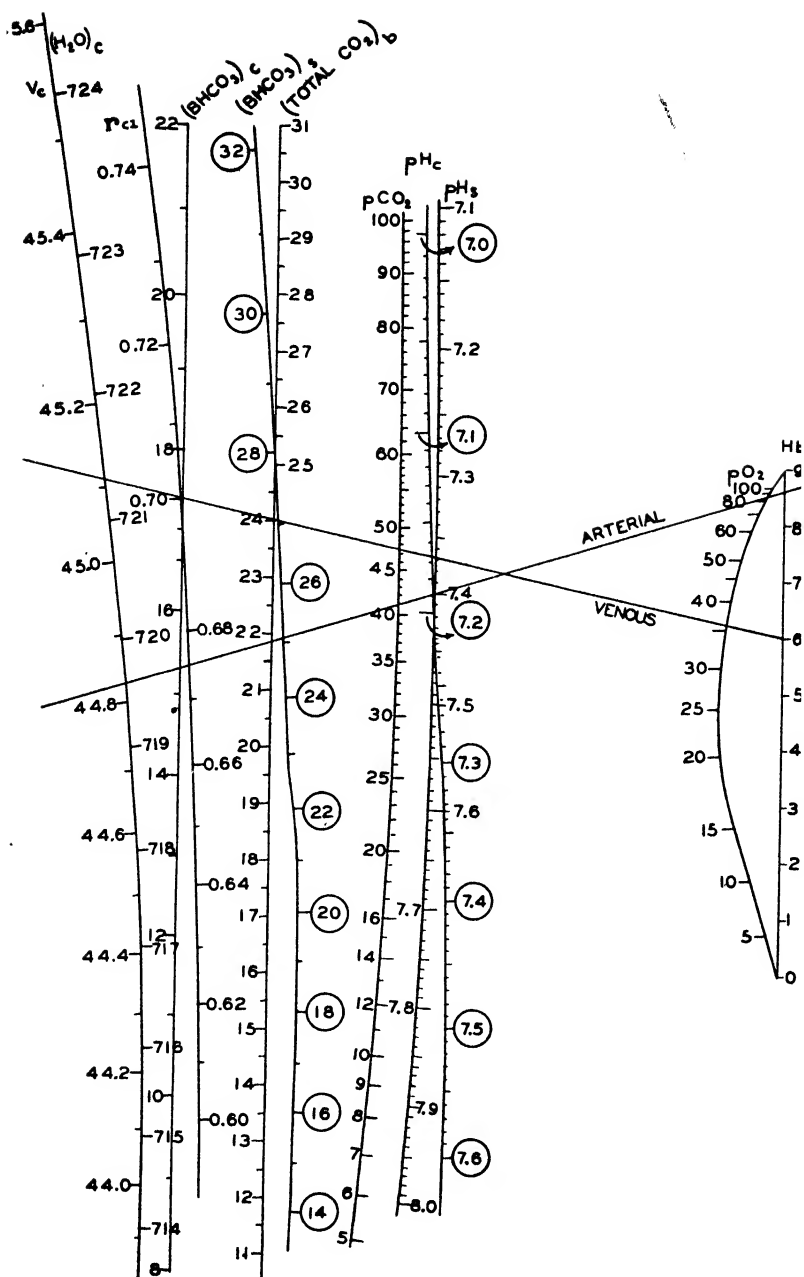
FIG. 6. Nomogram for human blood

TABLE II

Blood of Man in Respiratory Cycle

Concentration of hemoglobin, 9.00 mM per liter of blood; concentration of serum proteins, 39.8 gm. per liter of blood; respiratory quotient, 0.82.

	Arterial			Venous			Δ		
	Serum	Cells	Blood	Serum	Cells	Blood	Serum	Cells	Blood
H_2O, cc. per l. blood.........	517.5	322.8	840.0	514.7	325.6	840.0	-2.8	+2.8	0.0
B, m.-eq. " "	84.02	49.58	133.60	84.02	49.58	133.60	0.0	0.0	0.0
BX, " " "	2.08	-0.46	1.62	2.06	-0.33	1.73	-0.01	+0.12	+0.11
B lactate, m.-eq. per l. blood.	0.8	0.3	1.1	0.8	0.3	1.1	0.0	0.0	0.0
BCl, m.-eq. per l. blood.....	57.71	24.30	82.01	56.84	25.17	82.01	-0.88	+0.88	0.0
BP, " " " "	9.60	18.58	28.18	9.47	16.61	26.08	-0.13	-1.97	-2.10
$BHCO_3$, m.-eq. per l. blood..	13.83	6.86	20.69	14.85	7.83	22.68	+1.02	+0.97	+1.99
H_2CO_3, " " " "	0.71	0.48	1.19	0.81	0.56	1.37	+0.10	+0.08	+0.18
Total CO_2, m.-eq. per l. blood.	14.54	7.34	21.88	15.66	8.39	24.05	+1.12	+1.05	+2.17
$HbCO_2$, m.-eq. per l. blood..		1.09	1.09		1.72	1.72		+0.63	+0.63
Free O_2, mM..............			0.10			0.04			-0.06
Combined O_2, mM per l. blood..			8.60			6.01			-2.59
Total O_2, mM per l. blood...			8.70			6.05			-2.65
pCO_2, mm. Hg...........			41.0			47.5			+6.5
pO_2, " "			88.0			37.2			-50.8
Volume, cc. per l. blood....	551.7	448.3	1000.0	548.9	451.1	1000.0	-2.8	+2.8	0.0
pH...................	7.400	7.190		7.368	7.167		-0.032	-0.023	
r_{Cl}....................			0.675			0.700			+0.025
r_{HCO_3}.................			0.796			0.834			+0.038

$BHCO_3$ includes all forms of combined CO_2, including $HbCO_2$. Roughton (2) believes that in addition to bicarbonate and $HbCO_2$ there may be a third form of combined CO_2 in blood. It is due to the uncertainty regarding the quantitative relations among these three forms that all have been lumped together and for convenience designated $BHCO_3$. See the discussion of $HbCO_3$ in the text.

Since this paper was submitted, Stadie and O'Brien (20) have reported that about 3 per cent of the CO_2 in plasma is $HbCO_3$ and that the transport of CO_2 is 45 per cent as $HbCO_2$ in cells and 18 per cent in whole blood. According to Ferguson (4), the percentage of CO_2 transported as $HbCO_2$ is about one-half greater in cells and blood.

The concentrations of various substances in arterial and venous blood are given in Table II.   In Table II base bound by serum protein is calculated from the data of Van Slyke, Hastings, Hiller, and Sendroy (21).

$$BP_s = 0.104 \text{ (gm. protein) (pH}_s - 5.08) \tag{10}$$

The equations commonly used for calculating BP_c in oxygenated and reduced blood are those of Van Slyke, Wu, and Mc-Lean (14) derived from studies of solutions of horse hemoglobin. We have developed equations for human red cells based on the assumption that the pH_c of minimal base-binding is the same for the two species.   With the revised pK'_c used in this paper the pH_c of minimal base-binding is 6.63 in the oxygenated state and 6.70 in the reduced state.   The corresponding pH_s values are 6.628 and 6.756.   If 0.516 is subtracted from each of the values in Column 6 of Table I, Paper X, data are obtained for a titration curve of HbO_2 which passes through the point $pH_s = 6.628$ and $BP_c = 0$.   The following equation accords very closely with this titration curve.

$$BP_c = HbO_2 \left[-0.5 \text{ (pH}_c)^2 + 10.625 \text{ pH}_c - 48.46\right] \tag{11}$$

The equation similarly derived for reduced hemoglobin is

$$BP_c = Hb \left[-0.214 \text{ (pH}_c)^2 + 6.207 \text{ pH}_c - 31.97\right] \tag{12}$$

In these equations BP_c, the base bound by hemoglobin and other non-diffusible constituents of cells, is expressed in milliequivalents.   The concentration of oxyhemoglobin, HbO_2, is expressed in terms of mM of combined oxygen per liter of blood. The millimolar concentration of reduced hemoglobin, Hb, is the difference between the oxygen-combining capacity and the combined oxygen present.

The value given for base of serum in Table II is derived from the mean values given for Na, K, and Ca in Table I and an assumed value for Mg of 2 milli-equivalents per liter of serum. Cell base is taken as the sum of Na and K plus an allowance of 2 milli-equivalents for Mg.   BX is the difference between the sum of the four cations and the sum of determined anions.   Its value would be 0 if all cations and all anions were determined correctly.

It is not possible to represent $HbCO_2$ within the range of this

nomogram because of the peculiarity of its behavior; the smallest value for $HbCO_2$ in reduced blood exceeds the largest value in oxygenated blood. The concentrations shown in Table II are calculated from Ferguson's data (4). According to him, the transport of CO_2 in dilute hemolysate of human cells may be expressed by the relation $dHbCO_2/dHbO_2 = -0.24$. In the respiratory cycle with an oxygen transport of 2.65 mM, the transport of CO_2 as $HbCO_2$ is 0.63 milli-equivalent. Ferguson estimates that fully oxygenated hemolysate with the same oxygen capacity as normal blood contains 2 to 7 per cent of its CO_2 as $HbCO_2$. If 5 per cent is taken as a fair figure for arterial blood, its $HbCO_2$ content is 1.09 milli-equivalents and that of venous blood is 0.63 greater or 1.72 milli-equivalents. If these calculations are correct, the $HbCO_2$ content of arterial cells is 14.8 per cent of their total CO_2 and of venous cells, 20.5 per cent.

These estimates of $HbCO_2$ have been used to calculate the distribution of other forms of combined CO_2 between cells and serum. The distribution ratio in arterial blood for combined CO_2 other than $HbCO_2$ is the same as for the chloride ion, 0.67. In venous blood, however, the ratio is 0.62, which is much less than for chloride. If one assumes that 0.70, the chloride ratio for venous blood, also measures the distribution of non-carbamino-CO_2, the $HbCO_2$ content comes out 1.25 milli-equivalents. This represents only 15 per cent of the total CO_2 content of cells and provides for very little transport of carbamino-CO_2. If no mistake has been made in applying Ferguson's data on reduced hemolysate to partially reduced blood, the idea that the distribution of non-carbamino-CO_2 is the same as that of chloride is untenable. However, it must be borne in mind that the applicability of Ferguson's measurements to whole blood has not been tested experimentally. The opportunity which exists for dividing the transport of CO_2 between cells and serum introduces complications which do not exist in homogeneous hemolysate. Furthermore, it is conceivable that in the brief period of the respiratory cycle an equilibrium is not reached between serum and cells in regard to the various forms of combined CO_2. There exists the possibility that redistribution of ions between cells and plasma occurs after blood is drawn from the body even when escape of CO_2 is prevented.

SUMMARY

The distribution between cells and serum of combined CO_2 (Fig. 1) and of chloride (Fig. 2) has been described. The effect of oxygenation on the base-binding capacity of human hemoglobin has been measured (Fig. 4). Arterial blood has been described on the basis of samples from twelve men (Table I) and a synthetic method such as that developed by Henderson has been used to describe the relation between oxygenated and reduced blood (Fig. 6) and between arterial and venous blood (Table II).

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BLOOD AS A PHYSICOCHEMICAL SYSTEM

XII. MAN AT HIGH ALTITUDES

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It is the purpose of this paper to present data on electrolytes of blood at high altitudes and to develop a synthetic description of blood of man after he has become acclimatized to an altitude of 5.34 km. Detailed observations used in this synthesis have been published elsewhere in connection with other studies made by the high altitude expedition to Chile in 1935. Reference may be made to Talbott (1) for blood morphology; to Hall (2) and to Keys, Hall, and Barron (3) for measurements of affinity of hemoglobin for oxygen; to Dill, Christensen, and Edwards (4) for studies of gas equilibria in the lungs; to Talbott and Dill (5) for blood pictures in normal and sick residents; to Edwards (6) for blood lactate determinations; and to Forbes, Keys, and Hall (7) for pH measurements. Keys (8) has given a descriptive account of the expedition and made acknowledgments of grants in aid.

The nomenclature and chemical methods used are the same as in the preceding study, Paper XI. Serum[1] and cells of arterial blood treated with heparin were obtained by centrifugation in a No. 1 D.C. International centrifuge. A commercial power unit consisting of a gasoline engine and a D.C. generator of 250 watts capacity operated the centrifuge satisfactorily except at the highest laboratory station, 5.34 km. Here, on account of the cold and low oxygen, starting was difficult and the power output barely adequate.

After centrifuging under oil, the CO_2 content of serum was

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[1] The word "serum" in this paper is used to describe the fluid separated by centrifugation from blood treated with heparin.

determined.   The cells and serum were then transferred separately to ampules and a crystal of thymol added.   The ampules were sealed off and in this state the samples were kept as cold as possible until their analysis after our return to Boston.   Most
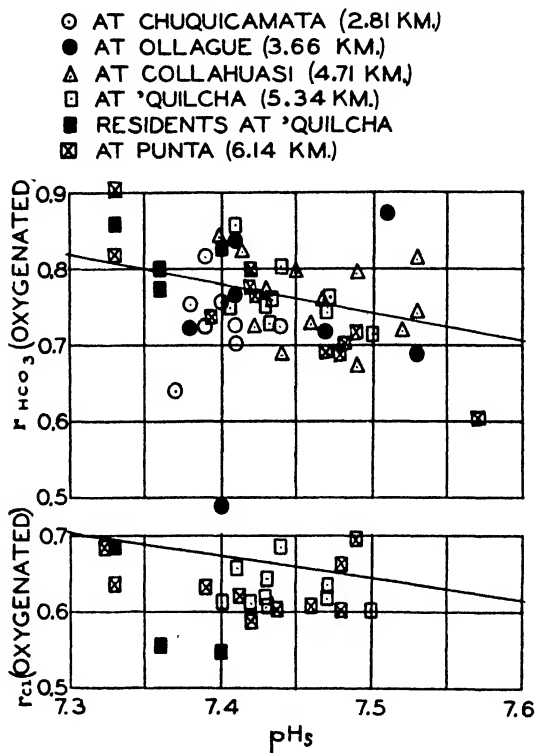
FIG. 1. The distribution of combined CO_2 and of chloride between cells and serum of oxygenated blood.   The observations were made on arterial blood without equilibration and calculated to the fully oxygenated state, the effects of oxygenation on distribution reported in Paper XI (9) being used.

samples reached Boston in good condition, although sampling of some was difficult because denaturation and precipitation of proteins had occurred.

Distribution of Electrolytes between Serum and Cells—The distribution ratio for combined CO_2, r_{HCO_3}, has been calculated from

measurements made in the field of cell volume and of total CO_2 in arterial blood and serum. Free CO_2 was estimated as described in Paper XI (9). By use of the relation of r_{HCO_3} to the degree of oxygenation, which was developed in Paper XI (9), the experimental values have been recalculated to the fully oxygenated state. In this form they have been plotted in relation to pH_s in Fig. 1. The points are almost evenly distributed about the line representing the normal value of r_{HCO_3} at sea-level. Aside from the influences of pH and oxygen saturation, the distribution of combined CO_2 is not appreciably changed at high altitudes.

TABLE I

Distribution of Combined CO_2 and Chloride in Oxygenated Blood at $pH_s = 7.40$

Station	Altitude	r_{HCO_3}		r_{Cl}	
		Observed	Deviation from standard	Observed	Deviation from standard
	km.				
Leadville	3.28	0.805	+0.022	0.0645	−0.029
"	4.60	0.837	+0.054		
Chuquicamata	2.81	0.731	−0.052		
Ollagüe	3.66	0.745	−0.038		
Collahuasi	4.70	0.785	+0.002		
'Quilcha	5.34	0.778	−0.005	0.0641	−0.033
" residents		0.792	+0.019	0.0651	−0.023
Punta	6.14	0.760	−0.023	0.0640	−0.034
Weighted mean		0.775	−0.008	0.0643	−0.031

The values for r_{Cl} are based on analyses of arterial serum and cells brought back to Boston. It appears probable from Fig. 1 that there is a slight reduction in r_{Cl} at high altitudes, since nearly all points fall below the standard curve.

All these observations in high altitudes have been recalculated to a pH_s of 7.40, the established relation between pH and the distribution coefficients (9) being used. The means for each station, found in Table I, confirm the impressions gained from the figures; i.e., r_{HCO_3} is unchanged and r_{Cl} is slightly less at high altitudes. The observed change of −0.03 in r_{Cl} disturbs but slightly the balance of electrolytes in serum and cells.

Proteins and Buffer Values—Our observations on serum protein, hemoglobin, and percentage of oxyhemoglobin in arterial blood
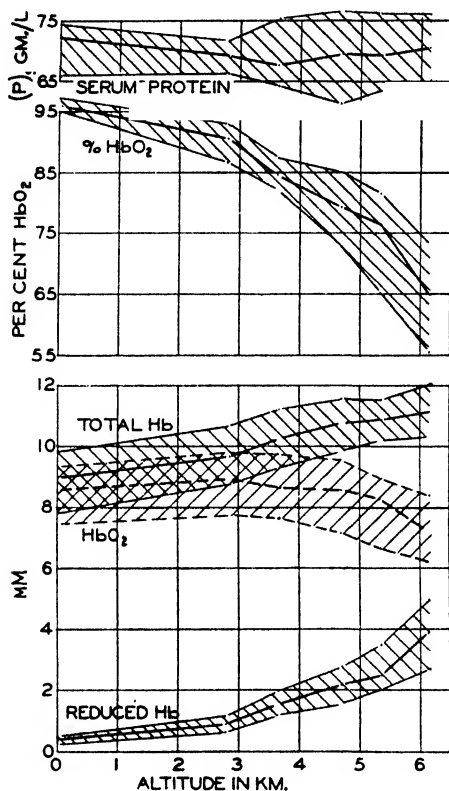
FIG. 2. Blood proteins in relation to altitude showing mean values on our group as well as the extreme range. Protein concentration in serum remains virtually constant while total Hb is increasing. Until the highest station is reached the increase in total Hb and reduction in percentage saturation of arterial blood with O_2 counterbalance so that the concentration of arterial HbO_2 remains constant. The increased range in percentage of HbO_2 at high altitudes depends on the properties of the oxygen dissociation curve; at a high oxygen saturation a change in pO_2 which produces little effect at high saturation produces a large effect at low saturation.

have been collected in Fig. 2. The protein concentration in serum does not change significantly. If blood volume remains constant as the proportion of red cells increases in high altitudes, the de

crease in the ratio of serum to cells means that the quantity of serum protein in the circulation diminishes. It is possible, however, that some increase in blood volume occurs. This seemed to be the case at least in the period of acclimatization both in the Pike's Peak party (10) and in three members of Barcroft's party to Peru (11). On the other hand, Margaria and Sapegno (12) found almost no change after 3 weeks at 2.9 km.

No important change was found by Talbott (1) in concentration of hemoglobin in red cells. It follows that one may apply to the blood of the high altitude resident the relationship which holds at sea-level.

$$V_s = 1 - 0.05 \text{ (total Hb)} \tag{1}$$

In this expression V_s is the volume of serum in 1 volume of blood and total Hb is measured by the mM of combined oxygen in blood saturated with oxygen.

Determination of total solids and of total nitrogen in red cells indicated that they were unchanged, not only in respect to hemoglobin but also to the total content of other organic substances. It is safe to infer that the buffer value of human red cells is the same at high altitudes as at sea-level.

The buffer system of blood is almost entirely dependent on protein; while the buffer value both of serum and of red cells remains constant, that of blood must increase in high altitudes because serum with 7 per cent protein is replaced by cells containing nearly 5 times the concentration of protein. A quantitative measure of the change in buffer value of oxygenated blood associated with this replacement may be developed from equations relating base-binding capacity to pH. For serum, according to Van Slyke, Hastings, Hiller, and Sendroy (13)

$$(BP)_s = (P)_s \, (0.104) \, (pH_s - 5.08) \tag{2}$$

In this equation $(BP)_s$ represents milli-equivalents of base bound by serum proteins and $(P)_s$ gm. of serum protein per liter of serum.

From the nomogram in Paper XI the relation of pH_s to pH_c in oxygenated blood between pH_s 7.2 and 7.6 is

$$dpH_s = 1.47 \, dpH_c \tag{3}$$

So long as pK' of serum and cells and the distribution of bicarbonate remain unchanged, this relationship of dpH_s to dpH_c remains applicable.

The buffer value of serum proteins in whole blood becomes by differentiation and substitution

$$\frac{d(BP)_s}{dpH_c} = (P)_s\, 0.153\, (1 - 0.05 \text{ total Hb})$$ (4)

Equation 11 of Paper XI (9) may be differentiated to give the buffer value of red cells of oxygenated blood.

$$\frac{d(BP)_c}{dpH_c} = (\text{total Hb}) [- pH_c + 10.625]$$ (5)

The buffer value of oxygenated whole blood, β_b, is the sum of the last two equations.   For most conditions it may be assumed that in arterial blood $(P)_s = 70$ gm. per liter of serum and $pH_c = 7.19$.   We can then derive a simple expression for the buffer value of blood.

$$\beta_b = \frac{d(BP)_s + d(BP)_c}{dpH_c} = 10.7 + 2.90 \,(\text{total Hb})$$ (6)

The magnitude of β_b is simply calculated.

	Total Hb	β_s
	$m\mathcal{M}$	
Serum alone.....................................	0	10.7
Blood at sea-level.............................	9	36.8
" after acclimatization at 5.34 km..........	13.5	49.8
Red cells alone...............................	20	68.7

The buffer value of red cells is about 7 times that of serum chiefly because of the greater concentration of protein in cells, but partially because, gm. for gm., oxyhemoglobin has about two-fifths greater buffering capacity than the usual mixture of serum proteins.

Alkaline Reserve—References so far made to buffer value have to do with the effects of addition or removal of acid in a closed system.   This is virtually what occurs in the respiratory cycle, since the rate of removal of CO_2 equals its rate of formation. When fixed acid in any quantity enters the body, another mechanism is called upon; excess CO_2 is eliminated by the lungs from the reserves of bicarbonate.   The total anion concentration in

serum may remain constant, the increase in new anion being balanced by decrease in bicarbonate with little or no change in pH. In respect to the first type of buffer action, blood at high altitudes has a greater β_b and the change in pH in the respiratory cycle is less than that of man at sea-level. In the second type it is inferior, since the alkaline reserve, or the concentration of CO_2 in arterial blood,[2] is much below that of normal man at sea-level.

The decrease in arterial CO_2 content with increasing altitude is shown in Fig. 3. This decrease is nearly proportional to the change in pCO_2 and as a consequence the pH variations are small. Roughly speaking, the relation between altitude and alkaline reserve is linear, but closer examination shows a large range in individual values and suggests that the decrease is small at first and greater for a given increase in altitude as higher altitudes are reached.

Time might be introduced as a third dimension in Fig. 3, but the small number of measurements do not warrant this. In our opinion, the subjects in Chile and in Colorado at 3 km. were almost fully acclimatized; if so, this part of the curve has reached its lowest value, assuming that the course of adaptation involves a decrease in alkaline reserve with time until equilibrium is reached. There are few records of measurements of alkaline reserve of human blood in altitudes between sea-level and 3 km. We doubt whether the 1st km. produces as much change as appears in Fig. 3. For altitudes above 3 km. our party as a whole was never fully acclimatized; the higher the altitude, the less complete the acclimatization. The proof of this comes from studies of the workmen at 5.34 km.; the mean alkaline reserve on six of them lies at the lower limit of the range in the ten members of our party at this station. It is probable that if months had been allowed for acclimatization at each station, the relation between

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[2] The alkaline reserve is often defined as the CO_2 content of oxygenated blood at $pCO_2 = 40$ mm. of Hg and sometimes as the CO_2 content of arterial blood. The definitions are approximately equivalent at sea-level but not at high altitude. Here, on account of the reduced and variable arterial CO_2 pressure, the CO_2 content of arterial blood gives a more significant measure of alkaline reserve than the content at a fixed CO_2 tension and percentage saturation with oxygen.

arterial CO_2 and altitude would have fallen on a curve showing an increased slope at the higher altitudes. If our observations at 3 km. in Leadville and in Chuquicamata and on workmen at 5.34 km. are taken as representative of complete-adaptation, the
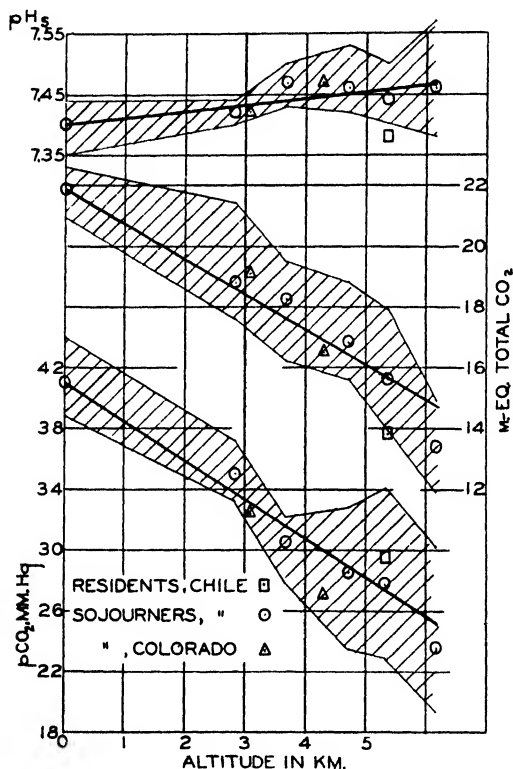
FIG. 3. Arterial pCO_2, total CO_2, and pH_s in relation to altitude. The heavy lines show the trend of the observations and the shaded areas, the extremes. The changes in pCO_2 and in total CO_2 are nearly proportional; pH changes only slightly. The observations in Colorado were made by Dill and his associates (14).

slope, $\Delta(CO_2)$ in milli-equivalent/Δ altitude in km., is 0.95 between 0 and 3 km. and 2.1 between 3 and 5.34 km.

It can now be stated with confidence that the pH of arterial blood increases little if at all up to 3 km. and that it is rarely higher than 7.5 up to 6 km. In support of this may be cited the

studies of Ewig and Hinsberg (15), who found an average arterial pH, of 7.40 on four men at 3.45 km. The earliest extensive

TABLE II

Electrolytes of Arterial Serum

The concentrations are given in milli-equivalents.

Subject	HCO₂	Cl	Lac-tate	Na	K	Ca		HCO₂	Cl	Lac-tate	Na	K	Ca
	At 2.81 km. (Chuquicamata)							At 3.66 km. (Ollagüe)					
B	21.8	107.0	1.7	140	5	4.9		23.2	105.5	1.1	143	3	5.2
C	22.0	106.7	1.6	141	5			22.0	106.5	1.9	138	5	5.5
D	23.8	104.7	1.5	139	6	4.9		23.5	104.9	1.2			
E	21.1	106.5	1.6	135	5	4.9		21.1	108.9	1.6			
F	21.9	105.1	1.6	140	6	5.3		23.4	107.2	1.0	141	5	5.0
H	22.4							20.2	106.1	4.0			
K	22.2		1.9					20.9	107.6	1.9	139	5	5.2
Mc	22.7	106.7	1.6	142		5.2							
M	22.0		1.8										
T	23.0	106.7	1.9	141		5.2		21.3	107.3	1.4	141	5	5.6
	At 4.70 km. (Collahuasi)							At 5.34 km. ('Quilcha)					
B	21.1	107.4	1.4	141	5	5.2		20.2	107.2	1.9	134	4	4.8
C	19.3	105.7	2.1	140	5			18.7	108.4	1.9	138	4	5.1
D	21.7	107.0	1.4	139	5	4.9		19.6	106.0	1.3	137	5	5.5
E	20.0	107.0	1.3	138	5	5.5		17.4	109.0	1.1	140	7	5.9
F	19.3	107.3	1.2	140	5	5.5		17.6	110.2	1.3	137	6	4.9
H	19.0	105.7	1.4	136	8	5.6		16.9	104.1	1.8	130	7	5.3
K	19.1	107.7	1.4	137	6	5.5		16.4	111.5	1.3	145	6	5.3
Mc	19.5		1.0					18.4	108.1	1.3	129	7	5.3
M	19.6	109.9	1.2	139	5	5.5		18.8	111.0	1.1	135	6	5.1
T	20.8	108.0	1.1	138	6	5.2		20.7	108.8	1.1	140	6	5.0

Subject	HCO₂	Cl	Lac-tate	Na	K	Ca	Subject	HCO₂	Cl	Lac-tate	Na	K	Ca
	At 6.14 km. (Punta)							'Quilcha workers					
B	17.5	106.8		142	4	5.3	An	18.3					
F	15.9	111.4	1.5	142	5	5.3	A	17.3	108.1	1.5	134	8	5.4
H	16.7	107.3					Bas	18.5	107.1	1.5	135	8	
K	14.6	109.9	1.4	134	7	5.3	Cam	17.0	108.3	1.3			
Mc	14.4	107.1	1.4	138	6	5.6	Fri	13.6	115.0	1.4		7	5.0
M	16.9	111.9	1.6	137	6	4.6	Her	17.9	104.9	1.8			
T	17.7	108.5	1.8	138	7								

measurements of pH at high altitude are those of Monge and associates (16). Twenty observations at 3.7 and 4.5 km. averaged 7.46 and only two were above 7.50.

Electrolytes of Serum—The complete record of our results on the principal ions of serum is found in Table II.   The cations and chloride were determined after our return to Boston.   Lactic acid was determined in whole blood and its concentration in serum estimated on the assumption that its distribution is the same as that of chloride.   Unpublished observations by Edwards of the Fatigue Laboratory support this assumption.

The individual response, with occasional exceptions, follows the pattern of the group taken as a whole.   The range in concentration for any given ion is somewhat greater than was found at sea-level (9).   The most complete results were at Collahuasi and 'Quilcha.   The extremes for each ion at these stations as com-

<div align="center">Table III</div>

<div align="center">*Range in Concentration of Ions Given in Milli-Equivalents*</div>

	Sea-level	Collahuasi	'Quilcha
HCO₃..............................	2.1	2.7	4.3
Cl................................	2.2	4.2	7.4
Lactate...........................	0.7	1.1	0.8
Na................................	11.6*	5	16
K.................................	3.7	3	3
Ca................................	0.8	0.7	1.0

* The large variations in Na and K at sea-level are due to the unusual concentrations found in the blood of one subject, Hall.   See Table I in Paper XI (9).

pared with sea-level are given in Table III.   The variability is increased slightly at 4.70 km. and considerably at 5.34 km.   It may be that technical difficulties at high altitudes had something to do with this increased variability.

Certain individuals maintain unusual characteristics at high altitudes.   Dill, for example, had the highest bicarbonate content at three of the four stations where his arterial blood was studied.   Hall was lowest in three out of five cases.   Hall had very low sodium and very high potassium concentration in his serum at sea-level and he maintained this characteristic at high altitudes.   Talbott's report (1) shows that Dill had the lowest hemoglobin content at sea-level and at every high altitude station.

Acid-Base Balance—The mean values for 4 anions including

proteinate and for 3 cations have been estimated for the group at sea-level and at each station (Table IV). Since magnesium nearly balances phosphate and sulfate, the sum of combined CO_2, chloride, proteinate, and lactate should nearly equal the sum of sodium, potassium, and calcium in the normal state. At sea-level the latter sum exceeds the former by 1.8 milli-equivalents and at higher altitudes by from 2.2 to 5.5 milli-equivalents. This increased excess of cations over anions may be due to a decrease in magnesium, to an increase in phosphate, sulfate, or some other anion, to unknown experimental errors, or to some combination

TABLE IV

Acid-Base Balance in Serum

The concentrations are given in milli-equivalents.

	Sea-level	2.81 km.	3.66 km.	4.70 km.	5.34 km.	6.14 km.	Miners at 5.34 km.
$BHCO_3$	25.1	22.3	21.9	19.9	18.5	16.2	17.1
BCl	104.6	106.2	106.3	107.3	108.4	109.0	108.7
BP	17.4	16.8	16.8	16.9	16.9	17.5	16.1
B lactate	1.4	1.7	1.5	1.4	1.4	1.5	1.5
Σ	148.5	147.0	146.5	145.5	145.2	144.2	143.4
Na	140.0	139.8	140.4	138.6	136.5	138.6	134.3
K	5.3	5.4	4.7	5.5	5.7	5.9	7.6
Ca	5.0	5.1	5.3	5.4	5.2	5.2	5.2
Σ	150.3	150.3	150.4	149.5	147.4	149.7	147.1
Δ	+1.8	+3.3	+3.9	+4.0	+2.2	+5.5	+3.7

of these factors. Excepting the highest station (where measurements were fewest) the discrepancy is of minor importance.

Table IV reveals that the considerable decrease in combined CO_2 is about one-half balanced by increase in chloride and about one-quarter by reduction in sodium concentration, leaving about one-quarter unexplained. Another notable feature of these results is the constancy of proteinate, lactate, potassium, calcium, and of the sum of the three cations. There may be some reduction in osmotic pressure, but it is very slight. Additional evidence on the latter question was obtained by carrying out on twelve sera returned to Boston measurements of osmotic

pressure.   These were made by C. Daly of our staff, using Hill's vapor pressure method (17).   The only two osmotic pressures outside the usual range were high.

Nomographic Description of Blood—The material so far presented has to do with the course of changes in blood during the process of adaptation.   For a more detailed description the synthetic method used for human blood at sea-level (9) has been employed.   It has been applied to blood of the 'Quilcha miners without modification, since it has been shown that the distribution of combined CO_2 is unchanged and it may be assumed that red cells and serum undergo no changes in buffer value.   The nomogram is shown in Fig. 4, and the table derived from it is Table V.   The arterial line is determined by analyses of arterial blood, and the venous line as shown is based on the assumptions of an R.Q. of 0.82 and an oxygen transport equal to that at sea-level.   The latter assumption is in accord with Grollman's study (18) and with unpublished observations made on our party by Christensen.

With this nomogram and Table V and with Fig. 6 and Table II of Paper XI (9) estimates can be made of the rôle of serum and cell proteins in CO_2 transport.   The CO_2 transported in the free state is about 8 per cent of the total in each case.   The remainder is transported as follows:

Combined CO_2 transport	Sea-level	5.34 km.
	per cent	*per cent*
By serum buffers.................................	6	4
" cell "	86	88

As a result of the chloride shift, the site of transport of combined CO_2 gives a different picture:

Site of combined CO_2 transport	Sea-level	5.34 km.
	per cent	*per cent*
Serum.......................................	47	33
Cells.......................................	45	59

The change in proportion is due chiefly to the change in cell volume per unit volume of whole blood.

We have estimated the $HbCO_2$ transport in the same way as
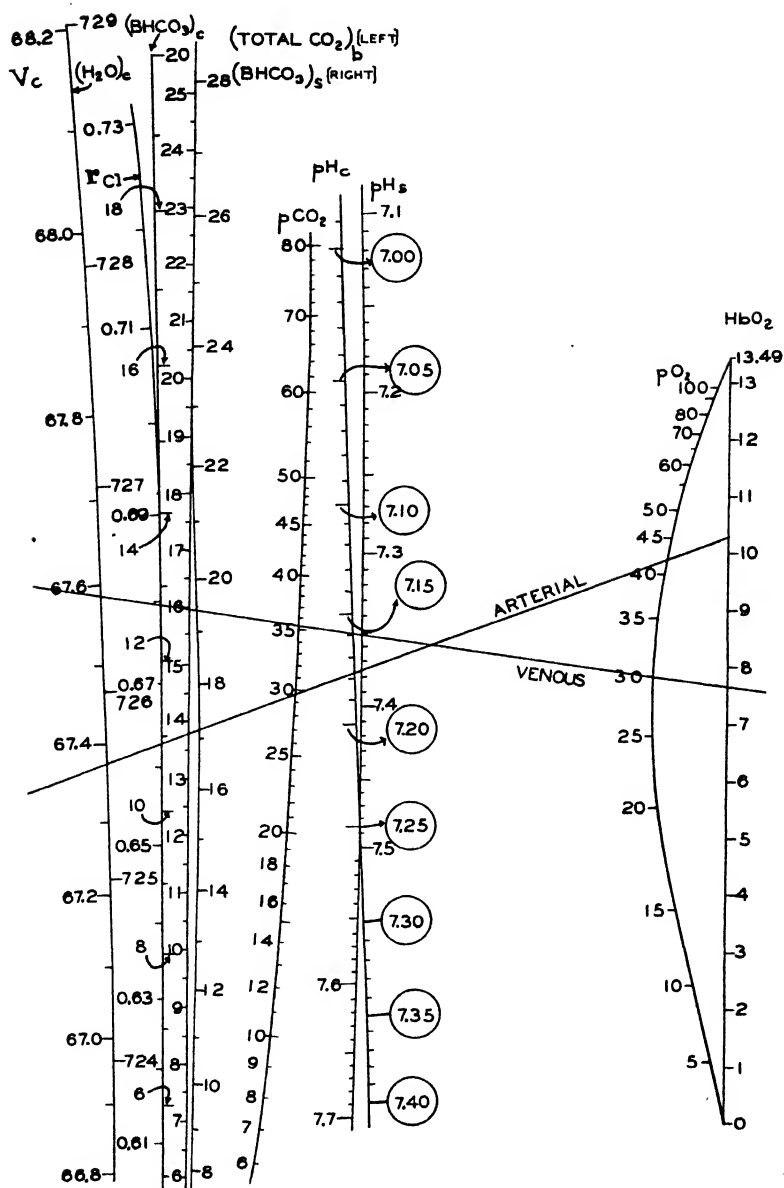
FIG. 4. Nomogram for blood of miners who reside at 5.34 km. and work at 5.8 km.

TABLE V

Blood of 'Quilcha Miners

Concentration of hemoglobin, 13.49 mM per liter of blood; concentration of serum proteins, 22.0 gm. per liter of blood; respiratory quotient, 0.82.

	Arterial			Venous			Δ		
	Serum	Cells	Blood	Serum	Cells	Blood	Serum	Cells	Blood
H_2O, cc. per l. blood	306.6	488.9	795.5	304.6	490.9	795.5	−2.0	+2.0	0.0
B, m.-eq. " " "	48.65	76.94	125.59	48.65	76.94	125.59	0.0	0.0	0.0
BX, " " "	1.82	5.59	7.41	1.81	5.82	7.63	−0.01	+0.23	+0.22
B lactate, m.-eq. per l. blood	0.5	0.5	1.0	0.5	0.5	1.0	0.0	0.0	0.0
BCl, m.-eq. per l. blood	35.47	37.50	72.97	34.84	38.13	72.97	−0.63	+0.63	0.0
BP, " " "	5.26	26.01	31.27	5.18	23.87	29.05	−0.08	−2.14	−2.22
$BHCO_3$, m.-eq. per l. blood	5.60	7.34	12.94	6.32	8.62	14.94	+0.72	+1.28	+2.00
H_2CO_3, " " "	0.30	0.52	0.82	0.36	0.63	0.99	+0.06	+0.11	+0.17
Total CO_2, m.-eq. per l. blood	5.90	7.86	13.76	6.68	9.25	15.93	+0.78	+1.39	+2.17
$HbCO_2$, m.-eq. per l. blood		0.69	0.69		1.32	1.32	0.0	+0.63	+0.63
Free O_2, mM			0.05			0.04			−0.01
Combined O_2, mM per l. blood			10.28			7.64			−2.64
Total O_2, mM per l. blood			10.33			7.68			−2.65
pCO_2, mm. Hg			29.3			35.3			+6.0
pO_2, " "			42.0			30.0			−12.0
Volume, cc. per l. blood	326.3	673.7	1000.0	324.3	675.7	1000.0	−2.0	+2.0	0.0
pH	7.376	7.178		7.352	7.159		−0.024	−0.019	0.0
r_{Cl}			0.663			0.679			+0.016
r_{HCO_3}			0.822			0.846			+0.024

for normal blood; on account of the increased proportion of hemoglobin and decreased alkaline reserve, there is doubt regarding the validity of this estimate.

The oxygen transport is carried on under strikingly different conditions. Arterial blood of the miner has a pO_2 less than one-half that of man at sea-level and yet its oxygen content is greater by one-fifth. The oxygen pressure in venous blood falls only 12 mm. below arterial in the first case and 50 mm. in man at sea-level. The miner has a venous pO_2 less by 7.2 mm. than that of man at sea-level. With a doubled transport of CO_2 and O_2 (such as occurs in moderate exercise) the conditions are as follows, assuming arterial blood is unchanged.

	Sea-level	5.34 km.
Venous HbO_2, mM	3.30	5.03
" pO_2, $mm.\ Hg$	21	21

It appears from this comparison that at 5.34 km. the fully acclimatized man with the same cardiac output as at sea-level maintains nearly as high a venous pO_2 in rest and possibly fully as high in moderate work. This is interesting in view of the fact that these men were able to climb each day from an altitude of 5.34 to 5.80 km. and carry out a day's work at the latter altitude.

The increased buffer value of blood at high altitude has been discussed above. Table V of this paper and Table II of Paper XI (9) make a quantitative comparison possible; ΔpH_c in the respiratory cycle, with CO_2 transport constant, is 0.023 at sea-level and 0.019 at 5.34 km. This indicates that the buffer value in the respiratory cycle is one-fifth greater; our previous calculation indicated that in oxygenated blood it is one-third greater; the difference is due to the fact that in the respiratory cycle part of the CO_2 is transported by the acid change in hemoglobin. For a given CO_2 transport this effect is almost independent of the amount of hemoglobin in blood.

The significant differences in electrolytes of whole blood between the miner and man at sea-level are given in Table VI. These changes depend in part on the change in proportion of red cells. If this were the only change occurring, the B and Cl would show slightly greater decreases than those observed. The

TABLE VI

Differences in Electrolytes of Whole Blood between Miner and Man at Sea-Level

The concentrations are given in milli-equivalents.

	B	Cl	HCO₃	Protein-ate	x
Man at sea-level..............	133.6	82.0	20.7	28.2	1.6
Miner at 5.34 km.............	125.6	73.0	12.9	31.3	7.4
Δ observed..................	−8.0	−9.0	−7.8	+3.1	+5.8
Δ if only V_c changes.........	−9.4	−11.4	−2.2	+5.4	−1.1

TABLE VII

Serum and Cells of Arterial Blood

	Standard man	'Quilcha miner	Δ
Serum			
H₂O, cc. per l. serum...........	938.0	939.6	+1.6
B, m.-eq. " " "	152.3	149.1	−3.2
BX, " " " "	3.8	5.6	+1.8
B lactate, m.-eq. per l. serum......	1.4	1.5	+0.1
BCl, m.-eq. per l. serum.........	104.6	108.7	+4.1
BP, " " " "	17.4	16.1	−1.3
BHCO₃, m.-eq. per l. serum........	25.1	17.2	−7.9
H₂CO₃, " " " "	1.3	0.9	−0.4
Total CO₂, m.-eq. per l. serum.....	26.4	18.1	−8.3
pH..............................	7.400	7.376	−0.024
Protein, gm. per l. serum..........	72.2	67.4	−4.8
Cells			
H₂O, cc. per l. cells..............	720.0	725.7	+5.7
B, m.-eq. " " "	110.6	114.2	+3.6
BX, " " " "	−1.0	8.3	+9.3
B lactate, m.-eq. per l. cells........	0.7	0.7	0.0
BCl, m.-eq. per l. cells............	54.2	55.7	+1.5
BP, " " " "	41.4	38.6	−2.8
BHCO₃, m.-eq. per l. cells..........	15.3	10.9	−4.4
H₂CO₃, " " " "	1.1	0.8	−0.3
Total CO₂, m.-eq. per l. cells.......	16.4	11.7	−4.7
HbCO₂, m.-eq. per l. cells..........	0.80	0.76	−0.04
Combined O₂, mM per l. cells......	19.2	15.3	−3.9
Total Hb, mM per l. cells.........	20.1	20.0	−0.1
pH..............................	7.190	7.178	−0.012

changes in these electrolytes are somewhat minimized by an increase in cell base and increase in serum chloride. The quantitative changes in serum and cells are as follows:

1. Loss of 3.2 m.-eq. base per liter serum
2. Gain " 3.6 " " " " cells
3. " " $\begin{cases} 4 & \text{"} & \text{Cl} \\ 2 & \text{"} & X \end{cases}$ " " serum
   Loss " $\begin{cases} 1 & \text{"} & \text{BP} \\ 8 & \text{"} & \text{HCO}_3 \end{cases}$ " " "
4. Gain " $\begin{cases} 1.5 & \text{"} & \text{Cl} \\ 9.3 & \text{"} & X \end{cases}$ " " cells
   Loss " $\begin{cases} 2.8 & \text{"} & \text{BP} \\ 4.4 & \text{"} & \text{HCO}_3 \end{cases}$ " "

A comparison of man at sea-level with the 'Quilcha miner in respect to composition of arterial serum and cells is made in Table VII. This table strengthens the impression that the changes in serum other than the reduction in bicarbonate and increase in chloride are small. The organism succeeds in maintaining stability in the face of extremely adverse conditions.

The writers are indebted to Professors L. J. Henderson, A. B. Hastings, and E. F. Adolph for many suggestions helpful in preparing this paper.

SUMMARY

The distribution of combined CO_2 between arterial cells and serum is unchanged at high altitudes aside from the responses due to changes in pH and per cent saturation with oxygen. There appears to be a decreased proportion of chloride in cells (Fig. 1). Owing to increase in the proportion of red cells, the buffer value of blood is increased; the pH, change in the respiratory cycle is one-fourth less. At the same time the decreased alkaline reserve reduces the capacity of the body to neutralize fixed acids. The increase in hemoglobin and decrease in alkaline reserve occur slowly; months may be required for hemoglobin to reach a maximum and arterial CO_2 content, a minimum.

The electrolytes of serum at high altitude were found to undergo one major change, whether measured in milli-equivalents or in per cent of the sea-level value. This is the decrease in bicarbonate. At 5.34 km. this may be 8 milli-equivalents, while the

Cl increase is 4 milli-equivalents and the Na decrease, 2 milli-equivalents, leaving an unexplained anion deficit of about 2 milli-equivalents.   Other ions are remarkable for their stability.

A synthetic description is given of the blood of fully adapted workmen living at 5.34 km. and working at 5.80 km.

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THE DIFFUSION COEFFICIENT OF INULIN AND OTHER SUBSTANCES OF INTEREST IN RENAL PHYSIOLOGY*

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An important question in renal physiology is the extent to which various urinary solutes may diffuse across the renal tubules. The possibility for such diffusion is especially great under the high concentration gradients that are established between the tubular urine and the blood by the reabsorption of water from the glomerular filtrate. The question is particularly important in the case of a substance like inulin which, on the basis of other physiological evidence, might be deemed suitable for the measurement of glomerular filtration.

Since little information was available on this question, we have determined the diffusion coefficients of inulin, creatinine, and other substances of physiological interest by the method of Northrop and Anson (1929).

In this method the solute diffuses across a thin, porous glass membrane, constituting the bottom of a closed cell, into water or solution contained in an outer jacket. This method has numerous advantages over the free interface method, since the diffusion front is mechanically stabilized. Our cells resembled the one depicted by Scherp (1933); they were 41 mm. in diameter and 20 mm. high, with a volume of approximately 20 cc. The bottom consisted of a Jena sintered glass membrane, 0.5 mm. thick, sealed to the cell with De Khotinsky cement. A stop-cock was inserted into the capillary stem 20 mm. above the cell. The outer jacket had an internal diameter of 50 mm. and a height of 170 mm. During operation three cells with their jackets were

mounted upon an aluminum triangular rack, the cell being mounted independently by the capillary tube. The clamps for mounting cell and jacket were so constructed as to permit independent adjustment in the vertical plane, and the rack carried thumbscrews at each corner which, in conjunction with variable contacts on the water bath, permitted adjustment to perfect horizontality. While an experiment was being set up, the rack was placed on a horizontal tripod. The cells were fastened to the rack, filled by gentle suction, washed with distilled water, and wiped with filter paper. Exactly 20 cc. of water were placed in each jacket, and the jackets were then fastened into place in such a position that, with the rack horizontal, perfect contact was obtained between the membranes and the surface of the water in the jackets. The rack was then transferred to the water bath, which was kept at $37° \pm 0.5°$ and its horizontality checked. At the conclusion of a diffusion period the water in the jacket was removed by aspiration and replaced with a fresh volume of 20 cc. of water at $37°$ by delivery into the open top of the jacket. The first diffusion period was analyzed and used in the calculation of the residual concentration of solute in the cell, but this diffusion period was not included in the determination of the diffusion coefficient. Three successive diffusion periods were then taken and calculated separately, and the results averaged. For all substances except Hb, for which the periods were lengthened to from 2 to 6 hours, the periods were about 60 minutes in duration.

Each membrane was calibrated by the use of 0.05 N sucrose in the presence of 100 mg. of creatinine per 100 cc. at $37°$ (for calculations see McBain and Liu (1931)). The diffusion coefficient of sucrose at $37°$ was determined as follows: six membranes were calibrated with 2.0 N NaCl at $25°$, with the value 1.246 per sq. cm. per day for the diffusion coefficient of this substance as determined from the temperature equation of Anson and Northrop (1937).[1] This figure checks with Clack's (1916–17, 1924). With these membranes the diffusion coefficient of sucrose at $25°$ was found to be 0.410 per sq. cm. per day without creatinine, and 0.418 in the presence of 100 mg. per cent of creatinine. This

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[1] We are indebted to Dr. Anson for making available to us his recent determinations of the diffusion coefficient of NaCl, prior to publication.

figure differs from McBain and Liu's (1931) value of 0.462 and from Öholm's (1929) value of 0.453 by about 10 per cent. The determination was repeated at 37° and, assuming no change in the cell constant, the diffusion coefficient of 0.05 N sucrose was found to be 0.552 without creatinine and 0.547 with creatinine. Öholm's (1929) figure for sucrose, when extrapolated to this concentration and temperature, is 0.583. Thereafter all cells were standardized at 37° with 0.05 N sucrose in the presence of creatinine, the diffusion coefficient of sucrose being taken as 0.55 per sq. cm. per day.

Creatinine was determined by the Jaffe reaction, with a photoelectric colorimeter; urea by urease decomposition and manometric CO_2 determination; sucrose and inulin by acid hydrolysis (0.1 N H_2SO_4) and the Folin (1929) sugar method; hemoglobin was read directly in a photoelectric colorimeter against a known hemoglobin solution; and chloride was determined by the method described by Smith (1930). The phenol red was dissolved in 0.01 M phosphate buffer of pH 7.4 and allowed to diffuse into this same buffer solution; the solutions were alkalinized with M Na_2CO_3 before colorimetric analysis. COHb was dissolved in 0.06 M phosphate buffer of pH 6.75 and allowed to diffuse into this same buffer solution.

The data given in Table I summarize our observations. Our primary intent in this investigation was to study different inulin preparations; and since a 1500 mg. per cent solution of this substance is supersaturated at 37°, it seemed possible that the inulin might deposit within the pores of the glass membrane and obstruct diffusion. For this reason and also to detect errors due to mechanical causes, defects, streaming, etc., creatinine (100 mg. per cent) was included in nearly all determinations. As will be seen in Table I, the diffusion coefficient of creatinine is increased by 0.05 N sucrose (and probably by urea), but it is unaffected by the presence of inulin (1500 mg. per cent). But creatinine has no apparent effect upon the diffusion of inulin itself. The routine inclusion of creatinine in the inulin determinations was valuable in that it showed that obstruction of the membrane by the inulin did not occur; otherwise the inclusion of a check substance appears to be superfluous.

A word may be said concerning the various inulin preparations

<div align="center">

TABLE I

Diffusion Coefficients at 37°

</div>

	No. of cells used*	Diffusion coefficient
		per sq. cm. per day
Urea, 2000 mg. %.............................	2	1.33
Creatinine, 100 mg. %........................	8	0.851
With sucrose, 0.015 N......................	1	0.836
" " 0.05 "......................	36	0.933†
" inulin, 1500 mg. %..................	39	0.846†
" urea, 2000 mg. %......................	2	0.915
Sucrose, 0.015 N.............................	3	0.558
" 0.05 ".............................	3	0.552
" 0.05 " with creatinine, 100 mg. %....	6	0.547‡
Phenol red, 32 mg. % with sucrose, 0.05 N......	3	0.537
Inulin (chicory), 1500 mg. %		
Sample 9.................................	2	0.203
Amorphous...............................	1	0.199
Sample 13................................	2	0.187
" 13 with 1% NaCl...................	2	0.189
" 13 " 1% " boiled 60 min.......	2	0.193
Inulin (chicory), 1500 mg. %. (All with creatinine, 100 mg. %)		
Sample 6.................................	3	0.202
" 7.................................	2	0.200
" 8.................................	3	0.204
" 9.................................	4	0.207
" 13.................................	2	0.195
		0.201 Average
" 9 boiled 30 min..................	3	0.195
" 13 " 30 "	2	0.195
" 13 " 60 "	2	0.202
" 13 " 120 "	2	0.199
Inulin (dahlia), 1500 mg. %. (All with creatinine, 100 mg. %)		
Sample 661-T.............................	2	0.203
" 661-NT............................	3	0.186
" 684-T	2	0.200
" 1226..............................	4	0.168
" RA................................	3	0.158
" RB................................	2	0.149
		0.177 Average
COHb, 2500 mg. %............................	6	0.086

* With three periods on each cell.

† Standard deviation, 0.033.

‡ Total of thirty-six periods.

which were examined. Certain samples of inulin produce marked reactions when administered intravenously to man in doses of 0.5 to 1.0 gm., whereas other samples may be administered in doses of 100 to 150 gm. without noticeable physiological effects (Shannon and Smith, 1935; Goldring and Smith, 1936).[2] The adverse effects of toxic samples are apparently attributable to particulate matter which can be removed by ultrafiltration (Cotui, McCloskey, Schrift, and Yates, 1937). Samples 6, 7, 8, 9, and 13 were chicory inulin[3] which was non-toxic for man in doses of 40 to 80 gm. Sample 661-NT was the non-toxic dahlia inulin used by Shannon and Smith (1935) in their original investigations, and produced no reaction in man in doses as large as 150 gm. Sample 1226 was dahlia inulin repurified by the manufacturer and produced no ill effects in doses of 100 gm. (cf. Goldring and Smith (1936)). Samples RA and RB were two preparations of dahlia inulin obtained from the Bureau of Standards and kindly supplied to us by Professor A. N. Richards. For Sample RA, which was purified by alcohol, the H_2O was 7.2 per cent, density 1.51, alcohol 0.01 per cent, and ash 0.05 per cent. For Sample RB, which was purified by recrystallization from water, the H_2O was 9.2 per cent, density (25°/4°) 1.48, ash 0.008 per cent, solubility in H_2O at 30° 0.185 per cent. Samples 661-T and 684-T were highly purified, yet very toxic preparations which had been obtained from the manufacturer. It was Sample 684-T from which Cotui et al. (1937) removed the toxicity by ultrafiltration. "Amorphous" inulin was prepared by pouring a hot concentrated solution of chicory inulin into a large volume of 95 per cent alcohol (Berner, 1931).

The native reducing power, total reducing power, and specific rotation of various samples of inulin, in comparison with the figures reported by others, are given in Tables II and III. There appears to be no difference between the toxic and non-toxic

preparations. Our data agree well with those found in the literature.

The samples of chicory inulin examined by us gave a slightly higher diffusion coefficient than dahlia inulin. Our figure on

TABLE II

Native and Total Reducing Power of Inulin

Sample No.	Source	Mg.-eq. glucose per 100 mg.	
		Native reducing power	Total reducing power
661-T............................	Dahlia	1.33	
661-NT...........................	"	1.50	
684-T............................	"	0.94	98.0
1226.............................	"	0.45	101.5
Yanovsky and Kingsbury (1933)...	"	0.81	
Bureau of Standards..............	Chicory		100.6
1................................	"	0.39	
3................................	"	0.37	
4................................	"	0.33	
6................................	"	0.40	100.6
7................................	"	0.42	
Yanovsky and Kingsbury..........	"	0.55	

TABLE III

Specific Rotation of Inulin

Sample No.	Source	Temperature	Per 100 cc. solution	$[\alpha]_D$
		°C.	gm.	degrees
684-T............................	Dahlia	25	4.56	−38.5
1226.............................	"	25	4.61	−39.4
Irvine and Steele (1920)..........	"	15	2.763	−34.2
Yanovsky and Kingsbury (1933)....	"	20		−36.9
Schlubach and Elsner (1930).......	"	25	3.09	−39.8
7................................	Chicory	25	4.725	−36.4
6................................	"	25	4.741	−36.3
Yanovsky and Kingsbury..........	"	20		−34.6
Pringsheim and Kohn (1924).......	"	20		−35.3

dahlia inulin (0.177 per sq. cm. per day) agrees with the value 0.186 obtained by extrapolating Öholm's (1929) data on inulin, with the same temperature coefficient as is shown by dextrin.

There seems to be no difference between the diffusion coefficient of toxic and non-toxic preparations. This is consonant with the belief of Cotui *et al.* (1937) that the toxicity is due to traces of contaminating ultramicroscopic particles. A small quantity of such a contaminant might impart considerable toxicity to the material without affecting the average diffusibility. It is not known whether the contaminant in toxic preparations is a carbohydrate or not. Goldring and Smith (1936) were able to remove part but not all of the toxicity by complete hydrolysis of inulin solutions, which would indicate that the contaminant was, perhaps, a refractory cellulose.

The molecular weight of highly purified dahlia inulin has recently been determined by Westfall and Landis (1936) by the thermoelectric vapor pressure method. These investigators obtain the figure 5100, which agrees well with the determinations of others, as cited by them. Haworth, Hirst, and Percival (1932) conclude that the inulin molecules are not in the form of large rings but consist of chains of fructofuranose, and have an average length of 30 units. From the reducing power they conclude that one terminal aldose group is free. There is every evidence from ebullioscopic (Drew and Haworth, 1928) and vapor pressure (Westfall and Landis, 1936) data to show that the polysaccharide readily undergoes partial hydrolysis on boiling, even in distilled water, but Drew and Haworth (1928) point out that dehydration may in certain instances tend to regenerate longer chain polysaccharides. It is especially interesting to note that prolonged boiling, either with or without NaCl, had no appreciable effect upon the diffusion coefficient of chicory inulin in our experiments. Neither have we found any marked increase in native reducing power after boiling. Such scission as occurs under these conditions may be compensated, so far as the average diffusion coefficient is concerned, by the opening up of polysaccharide rings into larger chains. In some instances we observed that boiling decreased the diffusion coefficient, a result consonant with the above suggestion. The point is an important one practically, because inulin solutions (5 to 20 per cent) in 1 per cent saline must be boiled for 5 minutes to effect sterilization before administration to man. From our results we believe that this procedure is without significant effect upon the average molecular size or

diffusibility.   This view is substantiated by the fact that during purification inulin is repeatedly boiled without evident degradation of the final product.

The solubility of inulin varies considerably with the mode of preparation.   Yanovsky and Kingsbury (1933) have shown that dahlia inulin is less soluble in cold water than chicory inulin; and each of these compounds exists in two modifications having different solubilities.   Both substances are more soluble in water if recrystallized from an alcohol-water mixture than if recrystallized from water.   The more soluble chicory inulin is unstable, gradually changing to the less soluble form.   The presence of levulose increases the solubility of dahlia, but not of chicory, inulin.   Both are readily soluble in hot water and form supersaturated solutions.   The adsorption of small quantities of alcohol, etc., apparently enhances solubility, and traces of non-aqueous solvents are difficult to remove (Berner, 1930).   When repurified from water, inulin consists largely of spherical crystals, but Berner (1931) has shown that an amorphous form, having the same molecular weight, is produced by pouring a hot, concentrated aqueous solution into a large volume of absolute alcohol, or by repurification from glycerol, acetamide, etc.   This amorphous preparation is readily soluble in cold water, but goes over to the insoluble form either in solution or a moist atmosphere.   The amorphous nature of alcohol-precipitated inulin has been confirmed by Katz and Weidinger (1931) by x-ray spectrum analysis. In our observations the amorphous preparation has the same diffusion coefficient as the crystalline material.

In the case of large spherical molecules it is possible to calculate the approximate molecular weight from the diffusion coefficient and the density by the Einstein equation (*cf.* Northrop and Anson (1929); Anson and Northrop (1937)).   Such a calculation applied to dahlia inulin (diffusion coefficient 0.177, density 1.50) gives the figure 15,360.   The discrepancy between the apparent molecular weight, as calculated from the diffusion coefficient, and the figure obtained by colligative methods (*i.e.*, 5100, which we may take as the true molecular weight) is unquestionably due to the molecular configuration.   Einstein's equation assumes that the molecule is spherical, and any departure from sphericity lowers the diffusion coefficient.   From the calculations of Sved-

berg (1928) and our diffusion coefficient it may be estimated that the inulin molecule is at least 10 times as large along one axis as along the other, confirming the conclusion of Haworth, Hirst, and Percival (1932) that the molecule is extremely elongate. This elongation has the effect, so far as diffusion is concerned, of increasing the apparent molecular weight. (Hydration of the molecule would have this same effect, but there is no evidence that the inulin molecule is hydrated.) We may say that, with respect to diffusion, inulin behaves as though it had a molecular weight of 15,360. In the same sense hemoglobin, which has a true molecular weight of 68,000, behaves as though it had a molecular weight of 117,000. This figure, based upon our diffusion coefficient, and a density of 1.33, agrees well with the estimate of 113,000 made by Svedberg and Nichols (*cf.* Nichols (1928); McBain and Liu (1931)) using the sedimentation velocity (*cf.* also Anson and Northrop (1937)).

The evidence of Hendrix, Westfall, and Richards (1936) on frogs and *Necturi* and of Shannon and Smith (1935), Shannon (1936), Richards, Westfall, and Bott (1936), and Kaplan and Smith (1935) on man, dogs, and rabbits indicates that inulin is completely filtrable through the glomerular membranes. On the other hand, hemoglobin is not readily excreted in the urine of mammals, presumably because the molecule is too large to pass through the glomerular membranes (Bayliss, Kerridge, and Russell, 1933). Since the diffusion coefficient of hemoglobin is about one-half that of inulin it would appear that for the purposes of measuring glomerular filtration little could be gained by seeking a molecule larger than inulin.

No comment is needed at this time on the diffusion coefficients of the other substances examined, except to note that our figure of 1.33 for urea is identical with the value obtained by extrapolating Öholm's (1929) data on 0.25 M urea to 37°. Similar extrapolations of the data given by this author for arabinose (0.1 N), maltose (0.1 N), lactose (0.1 N), dextrin (1.0 N and 0.1 N), and starch (1.25 per cent) would give, respectively, 0.89, 0.51, 0.53, 0.15, and 0.12. It is perhaps significant that the values for dextrin and starch lie within the critical range of what we have supposed to be the limiting permeability of the glomerular membranes. The data for gum arabic are uncertain because of the influence of salts on the diffusion coefficient (Bruins, 1931).

SUMMARY

Diffusion coefficients at 37° (per sq. cm. per day) have been determined for urea (1.33), creatinine (0.85), sucrose (0.55), phenol red (0.54), chicory inulin (0.201), dahlia inulin (0.177), and COHb (0.086).

Inulin has a smaller diffusion coefficient than would be expected from its molecular weight (5100), which fact is attributed to the elongate configuration of the polysaccharide molecule. So far as the diffusion rate is concerned, inulin behaves as though it had a molecular weight of over 15,000. Boiling in distilled water or in 1 per cent NaCl solution does not significantly increase the diffusion coefficient.

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INFLUENCE OF THE INORGANIC SALTS IN THE DIET ON THE COMPOSITION OF THE ASH OF CERTAIN TISSUES OF THE RAT*

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There has been ample demonstration that the consumption of a ration deficient in inorganic salts for relatively long periods results in changes in composition of tissues. Light *et al.* (1934) demonstrated the alterations in the composition of the entire body of the albino rat. The composition of the bone is changed (Brooke, Smith, and Smith, 1934), but with realimentation with an adequate ration the composition and strength rapidly approach the normal value (Clarke, Bassin, and Smith, 1936). Analysis of the kidney has shown that this organ is responsive to the metabolic demands imposed on the animal by lack of adequate salts in the experimental ration (Swanson, Storvick, and Smith, 1936). However, despite the dietary deficiency and perhaps because of the various functional adjustments to the metabolic emergency, the chemical composition of the blood remains largely unchanged (Smith and Smith, 1934). The present study extends the observations to the analysis of other tissues under the dietary conditions employed in the foregoing investigations as well as where certain ions are replaced in the deficient ration.

EXPERIMENTAL

Animals and Diets—Male albino rats from the Connecticut Agricultural Experiment Station strain, weighing 45 ± 4 gm. at

weaning (21 days old), were allowed free access to an adequate stock ration consisting of modified calf meal[1] (Maynard, 1930) and paste food,[2] supplemented daily with lettuce and yeast. If they attained a weight of 120 ± 4 gm. when they were 34 ± 2 days old, they were selected for the experiment.

The basal diet consisted per 100 gm. of casein[3] 18 gm., hydrogenated fat[4] 27 gm., dextrin[5] 55 gm. Vitamins were provided separately each day by 200 mg. of dried yeast, 5 drops of cod liver oil, 1 ml. of alcoholic extract of wheat germ, and 2 drops of wheat germ oil.

The appetite of the rat on the low salt diet is poorer than that of the normal rat. Its daily food consumption has been determined in previous investigations (Brooke and Smith, 1933; Clarke and Smith, 1935). In order to eliminate the influence of variations in intake of energy, protein, and vitamins, all animals of the present study were given each day the amount of food indicated by the average intake of the large number of rats on the low salt diet previously studied, according to the following schedule.

Period *days*	Basal diet per rat per day *gm.*
1– 7	7.1
8–14	6.4
15–35	5.7
36–60	5.6

The daily quantities of inorganic elements were for the most part based upon 408 mg. of Osborne and Mendel (1917) salt mixture, since this amount represents the average daily consumption of normal male rats eating *ad libitum* an adequate synthetic ration containing 4 per cent of the salt mixture, a quantity long considered adequate. Formulas were calculated for each diet and for each period in such a way that the desired amount of inorganic adjuvant was offered daily to each rat together with the desired amount of basal ration. Table I shows the various

[1] Modified by the addition of cod liver oil, 3 per cent.

[2] Whole milk powder, 25 per cent; casein, 25 per cent; wheat germ, 20 per cent; lard, 30 per cent.

[3] Described by Swanson and Smith (1932).

[4] Crisco.

[5] White, commercial.

groups, the mineral supplements, and the amounts of the different elements given daily together with the abbreviations by which the groups are hereafter designated.

The method insured a fair degree of uniformity in food consumption throughout the groups. As would be expected, however, some animals did not conform exactly to the schedule. These, when possible, were fed by hand. The animals were given redistilled water, and their caging and care followed closely the suggestions of Smith, Cowgill, and Croll (1925).

TABLE I

Inorganic Elements (in Mg.) Added to Diets

Group No.	Mineral supplement		Supplied daily					
	Description	Given daily	Ca	Mg	Na	K	Cl	P
I	Osborne-Mendel salt mixture*	408	50.3	7.2	15.4	80.0	21.9	40.9
II	Same minus NaCl and K†	408	99.3	7.2	1.7	7.2	3.9	44.5
III	Ca + P‡	152	50.3	0.2	1.4	7.0	3.9	40.9
IV	NaCl + K§	242	0.3	0.2	15.4	63.0	24.7	14.6
V	"	35.6	0.3	0.2	15.4	7.0	24.7	14.6
VI	Low salt		0.3	0.2	1.4	7.0	3.9	14.6

* Prepared as described by Osborne and Mendel (1917).

† Essentially the Osborne and Mendel salt mixture with sodium and potassium carbonates and hydrochloric acid omitted; calcium carbonate was increased to 238.1 gm.

‡ Calcium carbonate, 404.4 gm.; phosphoric acid, 309.6 gm., 85 per cent (180.9 ml. of sp. gr. 1.71).

§ Potassium citrate ($K_2C_6H_5O_7 \cdot H_2O$), 207.8 gm.; sodium chloride, 35.6 gm.

Since most of the changes produced by the restriction of dietary salts are evident after 60 days on the diet, the present experiments were terminated at that time. Under ether anesthesia the animals were bled from the abdominal aorta, and the tissues were quickly removed, trimmed, adhering fluid removed, placed in covered tared bottles, and weighed.

Analytical Methods

Ash—The tissues were dried to constant weight in a vacuum oven at 60–65° in carbon dioxide at a pressure of 20 mm. of mer-

cury. Approximately 1 gm. samples of the dried and powdered liver and muscle were ashed to constant weight in platinum in a muffle furnace at 520°. The entire dried kidney was similarly ashed.

Potassium and Sodium—The ash of 1 gm. samples of dried muscle tissue was dissolved in hydrochloric acid and transferred to a 50 ml. volumetric flask. A weighed amount of calcium hydroxide was dissolved in the acid solution of the ash. The phosphates were precipitated by making the solution alkaline to phenolphthalein with ammonia, and after dilution to volume they were removed by filtration through a dry, ashless filter. Aliquots were evaporated with sulfuric acid and ignited. Potassium and sodium were determined by Hald's (1933) modification of the methods of Shohl and Bennett (1928) and Butler and Tuthill (1931) respectively. Blanks including exactly the same amount of all reagents were run with each determination.

Chlorides—Approximately 0.5 gm. of finely divided ground muscle tissue was analyzed according to the method of Van Slyke (1923–24) after an alkaline digestion as described by Sunderman and Williams (1933). Because of lack of material, it was necessary to make the chloride determination on pooled samples of nearly equal quantities of mixed muscle tissue of two animals within a group.

Total Lipids—Approximately 2.5 gm. of finely ground, dried tissue were analyzed for lipids, according to the procedure described by Light *et al.* (1934).

Discussion of Methods

The methods used were in general satisfactory. In the determination of 0.5 to 0.8 mg. of sodium, recoveries were on the average 97 to 98 per cent. With 1 to 3 mg. of potassium, recoveries were 98 to 99 per cent; and with 0.037 milli-equivalent of chloride recoveries were 98 to 100 per cent.

Results

Ash—A comparison of the ash of liver, kidney, and muscle (Table II) shows the latter to be slightly but consistently poorer in ash throughout the groups; total or partial deprivation of minerals produced no change in the ash content of muscle. Per unit of water the liver was somewhat richer in inorganic matter

than were the other tissues studied, and the livers of the animals of Groups IV (NaCl + K) and V (NaCl) may have contained more ash than the livers of the other groups.   The kidneys of Group III (Ca + P) contained more ash than did those of the other groups.   Grossly, the kidneys of this group presented a granular appearance, and histological examination revealed extensive calcification (Eppright and Smith, 1937).   The kidneys of the

TABLE II

Ash Content (in Gm.) of Tissues

Group No.		Kidney		Liver		Muscle	
		Per 100 gm. fresh tissue	Per 100 gm. water	Per 100 gm. fresh tissue	Per 100 gm. water	Per 100 gm. fresh tissue	Per 100 gm. water
I (Osborne-Mendel salt mixture)	Maximum	1.48	2.01	1.43	2.08	1.33	1.77
	Mean	1.43	1.85	1.39	1.98	1.27	1.69
	Minimum	1.33	1.77	1.35	1.94	1.20	1.60
II (Osborne-Mendel salt mixture minus NaCl and K)	Maximum	1.46	1.92	1.41	2.05	1.36	1.75
	Mean	1.40	1.85	1.36	1.96	1.30	1.73
	Minimum	1.29	1.69	1.32	1.87	1.27	1.69
III (Ca + P)	Maximum	1.92	2.47				
	Mean	1.54	2.03				
	Minimum	1.36	1.79				
IV (NaCl + K)	Maximum	1.49	2.00	1.53	2.15	1.35	1.77
	Mean	1.36	1.76	1.47	2.06	1.28	1.69
	Minimum	1.09	1.29	1.43	1.92	1.14	1.52
V (NaCl)	Maximum	1.53	2.02	1.55	2.12	1.38	1.85
	Mean	1.44	1.89	1.42	2.02	1.32	1.76
	Minimum	1.28	1.09	1.32	1.87	1.27	1.68
VI (Low salt)	Maximum	1.49	1.96	1.43	2.04	1.40	1.89
	Mean	1.41	1.86	1.38	1.98	1.27	1.69
	Minimum	1.36	1.79	1.31	1.87	1.18	1.56

rats on the low salt diet did not contain an excessive amount of ash.   However, a progressive mineralization of this organ may occur with deprivation of salts, because, after 90 days on the diet, there is a higher percentage of ash than normal in the organ and calcium appears to be a prominent constituent of the ash (Swanson, Storvick, and Smith, 1936).

Potassium—The potassium content of mixed muscle taken from

TABLE III

Potassium Content of Muscle Tissue

Group I Osborne-Mendel salt mixture			Group II Osborne-Mendel salt mixture minus NaCl and K			Group IV NaCl + K			Group V NaCl			Group VI Low salt		
Ash	Water	Fresh tissue	Ash	Water	Fresh tissue	Ash	Water	Fresh tissue	Ash	Water	Fresh tissue	Ash	Water	Fresh tissue
per cent	m$_M$ per l.	mg. per 100 gm.	per cent	m$_M$ per l.	mg. per 100 gm.	per cent	m$_M$ per l.	mg. per 100 gm.	per cent	m$_M$ per l.	mg. per 100 gm.	per cent	m$_M$ per l.	mg. per 100 gm.
35.12	153.2	450.4	33.11	148.5	436.8	30.44	138.5	403.4	24.86	118.0	345.4	32.05	131.9	393.2
37.47	153.4	452.4	33.29	147.6	424.8	32.17	141.5	417.8	27.19	119.0	355.4	31.80	140.3	417.3
35.25	152.9	455.3	31.82	138.2	408.2	32.69	148.2	435.1	28.50	125.2	367.1	29.46	136.5	398.6
36.97	152.2	452.0	33.40	147.5	432.3	31.31	148.1	390.3	27.99	120.8	357.7	31.75	133.5	395.2
36.55	160.1	473.5	30.80	144.2	421.5	29.86	116.4	342.3	26.55	123.0	360.8	30.06	146.6	421.3
34.77	152.6	448.9	33.07	142.8	420.3	29.29	130.4	395.7	29.27	133.1	396.3	32.27	129.0	382.0
34.47	152.0	448.4										29.02	125.4	367.8
												29.61	123.2	363.7
35.8	153.7	454.4	32.56	144.8	423.9	30.96	137.1	397.4	27.39	123.1	363.4	30.75	133.3	392.4

the thighs of rats in Groups I, II, IV, V, and VI is given in Table III.   The results of the analyses of individual rats are shown.

The muscles of the rats of Group I (Osborne-Mendel salt mixture) consistently contained more potassium than was found in any other group.   The uniformity of the values within this group is striking, the variation with six of the rats not exceeding 1.4 mM per liter of water.   In Group II (Osborne-Mendel salt mixture minus NaCl and K) the amount of potassium in muscle was slightly but consistently smaller than in Group I.   The variations, somewhat greater than in Group I, were slight.   The dietary potassium of Group II, as shown by Table I, was greatly reduced.

With the replacement of more than two-thirds of the dietary potassium in Group IV (NaCl + K), together with sodium and chloride, the muscles were no richer in potassium than the muscles of the rats on the low salt diet.   Furthermore, the muscle potassium of the potassium-fed animals did not exceed the concentration of potassium in the muscles of the animals in Group II which received no potassium supplement whatever; in fact, in most cases it was less.   Muscle tissue was poorest in potassium when sodium chloride constituted the only mineral replacement (Group V).   Although the potassium ingested by the rats of this group was no less than that ingested by the rats on the low salt diet, the potassium of the muscle tissue was considerably diminished.   In the group on the low salt diet the potassium content of the muscle was variable, never, however, attaining the values of Group I, but rather resembling the values of Group IV (NaCl + K).

Sodium—Table IV shows the sodium content of muscle tissue of albino rats given diets containing different inorganic supplements.   As was the case with potassium, the sodium content of muscle tissue did not vary directly with the amount ingested.   The muscles of Group I (Osborne-Mendel salt mixture) contained less sodium than the muscles of Group VI (low salt), and sodium occurred in greater concentrations in the muscles of Groups IV and V than in Group I, despite the fact that all these groups were fed the same amount of sodium.   Notwithstanding, the largest amount of muscle sodium occurred in Group V, the sodium chloride-supplemented group.   It follows from a comparison of Groups IV and V that potassium added to the sodium supplement diminishes somewhat the level of the latter in muscle.

Chlorides—The results of the analyses of muscle tissue for chlorides are given in Table V. In contrast to sodium and potassium, the chloride content of the diet is apparently reflected more directly in muscle chlorides. In Groups I, IV, and V, all of which were given comparable amounts of chlorides, the level of this ion in muscles was strikingly similar, being 17.3, 16.7, and 17.3 mM per liter of water respectively. In the muscles of animals on the low salt diet (Group VI) chlorides averaged 13.5 mM per liter of muscle water, a value distinctly lower than that of Group I. The chloride

TABLE IV

Sodium Content of Muscle Tissue

Group I Osborne-Mendel salt mixture			Group II Osborne-Mendel salt mixture minus NaCl and K			Group IV NaCl + K			Group V NaCl			Group VI Low salt		
Ash	Water	Fresh tissue	Ash	Water	Fresh tissue	Ash	Water	Fresh tissue	Ash	Water	Fresh tissue	Ash	Water	Fresh tissue
per cent	mM per l.	mg. per 100 gm.	per cent	mM per l.	mg. per 100 gm.	per cent	mM per l.	mg. per 100 gm.	per cent	mM per l.	mg. per 100 gm.	per cent	mM per l.	mg. per 100 gm.
3.11	23.1	39.9	2.58	19.7	34.0	3.11	24.0	41 2	3.64	29.3	51.5	3.43	23.9	42.0
3.56	24.7	43.0	2.99	22.5	38.1	3.62	27.0	47.0	4.13	30.8	53.8	2.59	20.6	33.9
3.06	22.6	39.6	2.61	19.3	35.5	3.59	27.7	47.8	4.00	29.8	51.5	3.62	28.4	48.9
3.00	21.0	36.6	3.03	22.7	39.2	4.39	31.1	54.7	3.97	29.1	50.7	3.55	25.3	44.1
2.89	21.5	37.5	2 76	22.0	37.8	4.03	26.6	46.1	4.00	31.6	54.3	3.12	25.1	42.7
2.97	22.1	38.3	2.86	21.0	36.4	3.15	23.8	42.4	3.90	30.1	52.7	4.24	28.8	50.2
2.86	21.3	37.1										4.15	30.5	52.6
												3.81	26.9	46.8
3.06	22.3	38.8	2.80	21.2	36.5	3.64	26.7	46.5	3.94	30.1	52.4	3.56	26.1	45.1

concentration in the muscles of Group II (Osborne-Mendel salt mixture minus NaCl and K), likewise restricted with respect to chlorides, was also slightly low. It is of interest that the variations in chlorides did not follow the changes in base.

Lipids—The percentage composition of lipids in muscle, liver, and skin of animals on the low salt diet and in the replacement groups is shown in Table VI. The livers in each group contained more fat than was present in the skin or muscle. The tissues of Group VI (low salt) were generally poorer in lipids than the tissues of the animals given all of the salts (Group I). Deprivation of

salts altered most markedly the lipid content of skin and least, that of muscle.   The addition of sodium, chloride, and potassium to the low salt diet (Group IV) did not promote the deposition of fat.   The addition of calcium and phosphorus, however, sufficed to produce a concentration of lipids in tissues similar to, but in

TABLE V

Chloride Content of Muscle Tissue

Group I Osborne-Mendel salt mixture			Group II Osborne-Mendel salt mixture minus NaCl and K			Group IV NaCl + K			Group V NaCl			Group VI Low salt		
No. of rats	Water	Fresh tissue	No. of rats	Water	Fresh tissue	No. of rats	Water	Fresh tissue	No. of rats	Water	Fresh tissue	No. of rats	Water	Fresh tissue
	mM per l.	$mg.$ per 100 gm.		mM per l.	$mg.$ per 100 gm.		mM per l.	$mg.$ per 100 gm.		mM per l.	$mg.$ per 100 gm.		mM per l.	$mg.$ per 100 gm.
2	17.5	46.8	2	15.0	39.6	2	15.3	40.6	2	18.4	48.2	2	13.4	36.1
2	16.7	44.8	2	14.7	39.2	2	17.7	47.5	2	17.1	45.6	2	13.2	35.3
2	17.6	47.1	2	15.9	42.3	2	18 9	51.2	2	17.2	46.1	2	13.3	35.7
1·	18.0	48.0	1	16.0	42.7	6	16.2	43.6	1	15.7	42.2	1	14.3	38.4
1	17.3	46.2	1	14.6	38.8				1	17 6	47.3	1	14.0	37.2
	17.3	46.4		15.2	40.4		16.7	45.0		17.3	46.1		13.5	36.2

TABLE VI

Lipid Content (Measured in Per Cent) of Tissues

Group No.	Muscle		Liver		Skin	
	Dry matter	Fresh tissue	Dry matter	Fresh tissue	Dry matter	Fresh tissue
I (Osborne-Mendel salt mixture).......	14.9	3.68	21.4	5.38	16.5	4.07
III (Ca + P)......................	14.5	3.59	20.4	5.05	15.4	3.81
IV (NaCl + K)........................	14.4	3.47	17.2	4.14	7.9	1.90
VI (Low salt)....	13.3	3.25	16.8	4.11	8.5	2.08

every case slightly lower than, that produced when all the salts were given (Group I).

DISCUSSION

The results of the present study in general agree with the limited number of analyses of muscle tissue for sodium, potassium, and

chloride, which have been reported. According to Katz (1896) and Costantino (1911) muscle contains 0.23 to 0.47 per cent potassium. The fresh muscle of frog contains 0.34 per cent potassium (Mitchell and Wilson, 1921), and according to Harpuder (1931), the skeletal muscles of rats contain from 341 to 446 mg. per 100 gm. of fresh tissues, depending somewhat upon the dietary modifications.

The sodium content of muscle is ordinarily very low; its existence in muscle plasma has been questioned, the results obtained being explained by the quantities of sodium existing in the intercellular fluid and blood. According to Heubner (1931) the sodium in the muscle of the dog ranges from 0.05 to 0.09 per cent and of rabbits from 0.015 to 0.055 per cent. Harpuder (1931) reported a range of values from 10.0 to 64.3 mg. per cent in rats. The chloride content of muscle is so small that its existence in the sarcoplasm, like that of sodium, has been questioned. In the muscle of human beings, rabbits, and dogs the reported chloride concentrations range from 0.04 to 0.08 per cent (Heubner, 1931). If, however, the chlorides of blood and intercellular fluid are deducted, the remaining values may be not more than 0.02 per cent.

Evidence that dietary potassium *per se* influences its concentration in muscle is derived from two observations in the present study. First, the potassium content of the muscles of Group I (Osborne-Mendel) was greater than in Group II (Osborne-Mendel salt mixture minus NaCl and K). The animals of these two groups were essentially paired; they consumed the same amount of food daily for the 60 days of the experiment. Their diets differed mainly in that the former contained liberal amounts of potassium and sodium chloride, while the latter did not. Second, the potassium content of the muscles of Group IV (NaCl + K) was greater than that of Group V (NaCl), in which groups the only dietary difference consisted of the additional potassium in the former.

Other ions in the diet, however, influence the potassium composition of muscle more profoundly than does potassium itself. Liberal reinforcement of the diet with potassium, as in Group IV (NaCl + K), did not result in muscles especially rich in potassium; however, the addition of other elements, as in Group II, produced muscles of high potassium content. Since calcium is the predomi-

nating basic ion of Group II, it seems that this ion may, under the present experimental conditions, be particularly favorable to the maintenance of a normal potassium content in muscle. This effect of calcium may be correlated with its ability to maintain the animals in good nutritive condition (Eppright and Smith, 1937). It is known that the potassium of muscle varies with activity, tonus, and state of degeneration (Heubner, 1931). It has also been observed that, with muscles fatigued beyond the physiological limit, potassium diffuses out of the cells so rapidly that as much as half of their store may be lost in about 5 hours (Mitchell and Wilson, 1921). A progressive loss of excitability apparently accompanied the potassium depletion. It is possible that the influence of calcium on the potassium content of muscle may have a bearing on the ability of the cells to retain potassium; with lack of calcium the permeability of the cells may be so altered that normal retention of potassium in the cells is impossible.

Although dietary sodium may, in a measure, influence its own concentration in muscle, it is apparently not of primary importance inasmuch as the muscle tissue of the animals on the low salt diet generally contained more sodium than was found in Group I (Osborne-Mendel). Most striking was the observation that the amount of sodium in the muscles of animals given all the salts was small, while under less favorable dietary conditions it was consistently augmented. The presence of calcium and related elements in the diet (as in Group II) produced muscles with a small concentration of sodium, as compared to the normal. In the absence of calcium and its related elements (as in Groups IV, V, or VI) the sodium content of muscle was high, although in some of these groups there was no added dietary sodium and the amount in the diet was known to be very small. Sodium and potassium uninfluenced by other ions in the diet are mutually antagonistic. The potassium content of the muscles of Group V (NaCl) was less than that of Group VI (low salt). Conversely the sodium content of the muscles of potassium-supplemented animals is somewhat diminished (see Groups IV and V). Inspection of Table VII reveals that some mineral other than sodium or potassium is essential to the preservation of the normal ratio of these two elements in muscle tissue. From the present series of studies calcium appears to be the element involved. A reciprocal relationship of

tissue potassium and sodium has been observed by others.   Gerard (1912) noted that the tissues of most active function had the highest potassium to sodium ratios whether they were muscular, glandular, or nervous.   He also obtained results indicating that the base content of the tissue cells can be influenced appreciably by that of the diet.   However, the apparent relationship of calcium to the preservation of the potassium to sodium ratio has not been reported heretofore, so far as we know.

This increase of sodium in muscle may imply increased sodium in serum, in extracellular fluid, or in muscle plasma.   Since the electrolyte content of the blood is normally remarkably constant

TABLE VII

Molecular Ratio of Potassium to Sodium per Liter of Muscle Water

Group I Osborne-Mendel salt mixture	Group II Osborne-Mendel salt mixture minus NaCl and K	Group IV NaCl + K	Group V NaCl	Group VI Low salt
6.6	7.5	5.7	4.0	5.5
6.2	6.5	5.2	3.8	6.8
6.7	7.1	5.3	4.2	4.8
7.2	6.4	4.7	4.1	5.2
7.4	6.5	4.3	3.8	5.8
6.9	6.8	5.4	4.4	4.4
7.1				4.1
				4.5
6.8	6.8	5.1	4.0	5.1

despite deprivation of salts (Smith and Smith, 1934) and since the cells are assumed to be relatively impermeable to sodium, it seems probable that increased sodium in muscles is a reflection of increased extracellular fluid.   However, since the sodium increased without a proportionate increase in chloride, which is the chief anion of extracellular fluid, it seems more likely that the sodium has actually entered the cell and replaced the potassium in muscle tissue.   The influence of dietary salts on tissue hydration will be discussed later.

Although the number of chloride analyses was small, they strongly suggest that dietary adjustments of chlorides are directly reflected in the chloride content of muscle.   In contrast to the

situation as concerns sodium and potassium, chloride storage in muscle is less markedly influenced by other ions. Moreover, although the chloride ion is biochemically related to the sodium ion, it does not, under these conditions, appear to vary directly with it in muscle tissue.

Although the limited number of analyses in the present series and the overlapping of results prevent generalizations, it seems that the total ash of the soft tissues is strikingly less susceptible to dietary alterations than is the ash of the skeletal system.

On autopsy marked absence in depot fat was observed, particularly in rats deprived of calcium and phosphorus. Actual determinations of lipids confirmed these gross ·observations and showed that the lack of lipids was not confined to the large depots, inasmuch as the tissues examined had been trimmed of all visible fat. In the deposition of lipids, calcium and phosphorus again seem to play a prominent rôle. The striking lack of lipids in the skin may be of interest in several connections. Loss of hair was particularly common in those groups in which lipids were most scarce. Since subcutaneous fat serves to protect against the loss of heat, the lack of lipids in the skin may bear a causal relationship to the fact that body temperature is lowered in the rat on the low salt diet. There may also be a relationship between the increased basal metabolic rate of the rat on the low salt diet (Kriss and Smith, 1935) and its impoverishment in lipids.

SUMMARY

The potassium and sodium contents of muscle are influenced more by the calcium in the diet than by the potassium and sodium included therein. The ratio of potassium to sodium in muscle tissues, much distorted on the sodium chloride, or sodium chloride plus potassium régimes, is quite normal when calcium is abundantly supplied, even though the diet is poor in these elements themselves. Potassium and sodium are mutually antagonistic with respect to their retention in muscle; diminished potassium and augmented sodium, in general, accompany a poor nutritional state. The chloride content of muscle does not follow these shifts of base, but varies more directly with the level of chloride in the diet. Directly or indirectly, removal of calcium and phos-

phorus from the diet greatly reduces the lipids in tissues, particularly in skin.

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FURTHER STUDIES ON THE CONCENTRATION OF THE ANTIPELLAGRA FACTOR*

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The non-identity of the true antipellagra factor and flavin is now quite definitely established. Using pellagra-like symptoms in chicks as a measure of antipellagric activity, we demonstrated, 2 years ago (1), that flavin was completely inactive but that liver extract retained its potency after the flavin was removed by adsorption on fullers' earth. At that time, there was some question, of course, whether the chick method gave a true assay of the human antipellagra factor. We supplemented our chick work, therefore, with studies on black tongue in dogs. Again we (2) found that preparations of flavin from liver extract were completely inactive in the prevention or cure of black tongue and that the filtrate from which the flavin had been removed was highly active. In a paper by Booher and Hansmann (3) which appeared after our paper had been submitted for publication, the following

This factor has been designated vitamin B_2 or G (B_2) in our previous papers (1, 2). However, owing to the fact that the definition of vitamin G (B_2) given by the Committee on Vitamin B Nomenclature of the American Society of Biological Chemists is confusing in light of our present knowledge, and because certain workers are using vitamin B_2 (G) for flavin, and the English workers retain the term vitamin B_2 for the whole of the more heat-stable part of the vitamin B complex, it seems best to refer to the factor described in this paper merely as the antipellagra vitamin. We still feel that it is most logical to use vitamin G for the antipellagra factor, but final decision must rest with the Committee on Nomenclature.

conclusion is made, "a vitamin G concentrate obtained from low-lactose whey powder which carries, in addition to vitamin G (lactoflavin) at least one other heat-stable vitamin necessary for rat growth, has been found effective for the prevention or cure of black tongue." Obviously, the high dose of the concentrate which they fed contained sufficient quantities of the antipellagra factor to cure the black tongue. Birch, György, and Harris (4) have verified the conclusion that pure lactoflavin has no curative action on canine black tongue.

Dann (5), working with human pellagrins, has shown that lactoflavin is inactive in the treatment of pellagra and that, when these patients were subsequently administered liver extract, remission of symptoms resulted. Thus there is unconflicting evidence that flavin is not the antipellagra factor and that another substance occurring in liver extract is responsible for the prevention and cure of pellagra.

In the past, most of the studies on vitamin B_2 or G have been carried out with rats. The condition which Goldberger and his associates produced in rats and which they believed to be analogous to human pellagra and canine black tongue appears, according to our present knowledge, to have been caused by the lack of other factors in addition to the antipellagra factor. In a recent publication by Richardson and Hogan (6) evidence is presented to show that the deficiency which Goldberger and Lillie (7) produced was due to a lack of flavin. Other attempts to produce pellagra in rats have resulted in vitamin B_6 deficiency. Very few, if any, investigators have produced uncomplicated pellagra in rats. A careful study of the description given by Goldberger and Lillie (7) of their animals indicates that the rats may have been suffering from a deficiency of at least three factors, e.g. flavin, vitamin B_6, and the antipellagra factor. In one or two animals they describe inflammation of the anterior part of the floor of the mouth and also diarrhea. They stated, at that time, that the possibility is not excluded that there may be in yeast more than one such thermostable factor which further study may succeed in differentiating. The difficulty has been in supplying all of the necessary factors for the growth of rats without contamination with the antipellagra factor. We (8) have recently found that rats need a new factor which is distinct from the known factors in the B

complex. We have designated it the alcohol-ether precipitate factor, since it can be separated from liver extract by precipitation with a mixture of ethyl alcohol and ether. A survey of the literature indicates that many of the rations which have been used for the production of pellagra must be low in this factor. We are now attempting to purify this factor, as well as other vitamins, to such an extent that rations very low in the antipellagric factor, yet complete in other respects, can be prepared for rats.

In the meantime, we have continued our studies on the isolation of the antipellagra factor using chicks and dogs for assay.

<div align="center">EXPERIMENTAL</div>

The concentrate used in the following procedure was the amyl alcohol fraction, the preparation of which has been described previously (1). The amyl alcohol solution equivalent to 400 gm. of liver extract was concentrated to dryness *in vacuo* at 50°. The residue was dissolved in 100 cc. of 95 per cent ethyl alcohol, transferred to a crystallizing dish, and the alcohol removed in a sulfuric acid desiccator at 65°. The residue was extracted with acetone and the insoluble matter filtered off, which was inactive. The filtrate was again concentrated to dryness and desiccated *in vacuo* over calcium chloride for 24 hours. The residue was extracted with acetone and more insoluble matter filtered off, which was inactive. After concentration to dryness and thorough desiccation, the solid matter was extracted with 50 cc. of water, whereupon much of the inert matter remained undissolved, which was filtered off and washed. The combined filtrate and washings were diluted to 200 cc. and shaken with 2.0 gm. of norit. The norit was filtered off and thoroughly washed with water. The combined filtrate and washings were colorless and contained 2.56 gm. of solid matter from 400 gm. of liver extract.

The assay of the final concentrate with chicks is shown in Table I. The chicks in the group receiving the basal Ration 240-H all showed pellagra-like symptoms at 3 weeks of age. The rate of growth was definitely retarded. The group receiving the concentrate equivalent to 5 per cent of the original liver extract, or about 0.7 mg. daily per chick, showed no lesions and weighed 200 gm. at 6 weeks of age. Thus at least 40 per cent of the vitamin present in the original liver extract was recovered in the

concentrate, since 2 per cent liver extract is required to protect chicks on this ration.

TABLE I

Records of Chicks Receiving Basal Ration 240-H Alone and Basal Ration Plus Vitamin Concentrate Equivalent to 5 Per Cent Liver Extract or Approximately 0.7 Mg. per Chick per Day

Four chicks were used for each experiment.

Ration No.	Cases of pellagra	Time of appearance	No. of chicks surviving	Weight at 6 wks.
		wks.		*gm.*
240-H................................	4	3	3	133
240-H + vitamin B₂ concentrate......	0		4	200
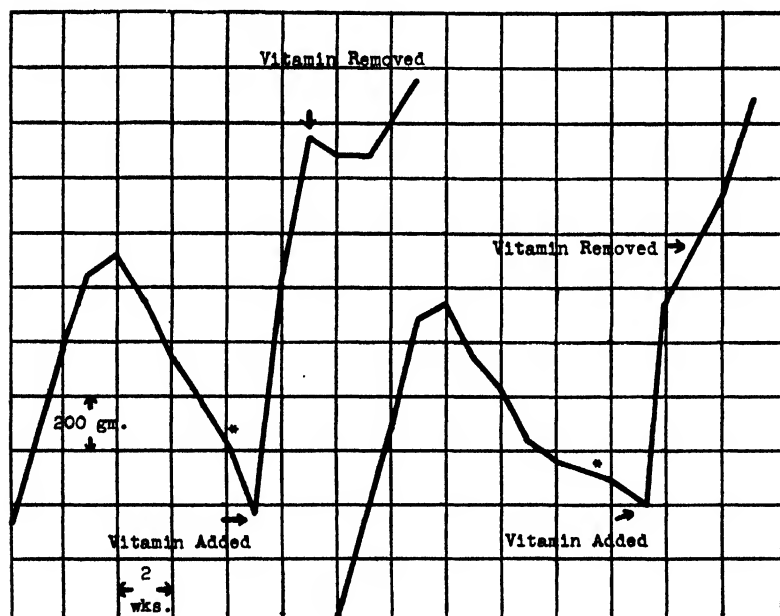
CHART I. Growth records showing the effect of the administration of 64 mg. of the vitamin concentrate daily to dogs suffering from severe black tongue. The asterisk indicates the appearance of severe black tongue.

This same concentrate was tested on dogs suffering from black tongue. The basal ration was essentially Diet 323 of Goldberger,

Wheeler, Rogers, and Sebrell (9), except that the cow-peas were omitted and the calcium supplied as both calcium carbonate and calcium phosphate. It had the following composition.

Yellow corn... 72
Purified casein... 18
Cottonseed oil.. 5
Cod liver oil... 2
Calcium carbonate.. 1
    "   phosphate....................................... 1
NaCl 1

Dogs were placed on this ration shortly after weaning until they developed typical symptoms of black tongue. This generally took 6 to 8 weeks. At this time the preparation to be tested was added to the ration. Since the dogs refused to eat when they were suffering from black tongue, the first dose was always fed by pipette. After the first treatment, their appetites improved immediately and the preparation was then added to a small amount of the ration each morning. In Chart I are plotted the growth curves of two dogs that received 10 cc. of the concentrate containing 64 mg. of dry matter daily. Remarkable responses occurred in both cases. Dogs which had refused food for several days began to eat 2 hours after administration of the concentrate. Within a day the dogs regained much of their vigor, and in 3 days they appeared quite normal. A very rapid increase in weight also resulted. Attempts have been made to purify the above concentrate further, but the assays have not been completed.

DISCUSSION

Lepkovsky and Jukes (10) have recently reported studies on the above factor. The properties of this factor which they describe check very well with those which we have found. In another paper on the distribution of this factor, Jukes and Lepkovsky (11) suggest that this vitamin should be called the "filtrate factor" because they think it is not identical with the P-P factor. However, the only evidence which they present is that wheat germ, which was found to be a good source of the antipellagra factor by Sebrell, is not highly active in preventing the pellagra-like lesion in chicks. Different samples of wheat germ may vary considerably in antipellagric activity. Although we must not

overlook the possibility that there may be two closely related factors, one active in chicks and one in humans, the fact that a purified concentrate cures both chick pellagra and canine black tongue is strong evidence that we are dealing with one factor.

Several investigators have mentioned the fact that the factor which we have been studying cannot be vitamin B_2 (G) or the P-P factor, because Goldberger and Lillie (7) reported that the antipellagra factor, or the factor which promoted growth and caused the disappearance of the pellagra-like condition in rats, was adsorbed on fullers' earth. We now know that flavin is adsorbed on fullers' earth and may have been the active material which these workers were dealing with. Goldberger also found that the limiting factor for growth in rats on a diet in which the supply of water-soluble B was Seidell's "activated solid," was the P-P factor. It is entirely possible that the fullers' earth as prepared by them carried some antipellagra factor, because they adsorbed directly from yeast extract and did not wash their adsorbate. We also know that the alcohol-ether precipitate factor is adsorbed, and it has very decided effect on the growth of rats. We have shown that the antipellagra factor obtained from liver extract is not adsorbed on fullers' earth and that a solution of the factor can be decolorized by charcoal without appreciably lowering its activity. Lepkovsky and Jukes also showed that it is not adsorbed on charcoal. Thus there is ample evidence that the antipellagric factor as found in liver is not adsorbed on colloids.

If we are correct in concluding that canine black tongue and human pellagra are identical, a conclusion which most workers agree to, the concentrate which produced such rapid cure of black tongue should be highly effective in the treatment of human pellagra.

SUMMARY

1. A method is described for the further purification of a concentrate of the antipellagric vitamin.

2. From 400 gm. of liver extract 2.56 gm. of active dry matter were obtained.

3. Dogs suffering from canine black tongue were completely cured when administered this concentrate at a 64 mg. daily level.

4. The concentrate prevented pellagra-like symptoms in chicks

when fed at a level equivalent to 5 per cent liver extract or about 0.7 mg. daily per chick.

    5. The antipellagric vitamin is not adsorbed on charcoal.

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THE DETERMINATION OF PHOSPHOLIPIDS IN BOVINE BLOOD

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In 1934 Blackwood (1) reported results indicating that there was no decrease in the lipoid phosphorus content of the blood in passing through the lactating gland of the cow, in opposition to the ·earlier work of Meigs and his associates from which it had been concluded that phospholipids were the source of milk fat. Data in agreement with those of Blackwood were obtained in this laboratory, but it was felt that the results would be more convincing if the phospholipids themselves were determined instead of being calculated from lipoid phosphorus. Accordingly, a study of the blood before and after passing through the gland was undertaken by the oxidative procedure of Bloor (2). Repeatedly the results thus obtained for phospholipid were not only variable but also 50 per cent or more below the values produced by the lipoid phosphorus method. This led us to a critical, comparative study of the two methods, which has ascertained the cause of the divergence of previous data. A modification of the procedure for isolating the phospholipids for oxidation has been developed by means of which the results obtained closely approximate those calculated from the determination of lipoid phosphorus. These studies are here described.

Preliminary Experiments

The Bloor method of isolating the phospholipids for oxidation was checked by determining the phosphorus in the various fractions obtained. First, the phosphorus in the alcohol-ether extract (lipoid phosphorus) was determined. After a suitable aliquot of this extract had been evaporated to dryness on the steam bath and the dry material extracted with petroleum ether, the phos-.

phorus in the ether extract and in the residue insoluble in the petroleum ether was determined. Next the petroleum ether extract was concentrated to a small volume and the phospholipids precipitated with acetone and $MgCl_2$. The precipitate was dissolved in moist ethyl ether or chloroform, and a phosphorus determination made here as well as on the acetone-soluble fraction.

It was found that the petroleum ether extract contained only a fraction of the phosphorus found in the alcohol-ether extract, the major part remaining in the residue. The acetone-soluble fraction contained a small and relatively constant amount of the element. The phosphorus precipitated by acetone and redissolved in the moist ether or chloroform solution was but slightly less than the total phosphorus in the petroleum ether extract, indicating that the residue of acetone precipitate insoluble in moist ether or chloroform contained very little if any phospholipid. The amount of phospholipid found in the moist ether or chloroform solution by the oxidative procedure agreed very well with the value obtained by multiplying the phosphorus present by the factor 25. Thus it became evident that the low results obtained by the oxidative procedure were caused either by a faulty petroleum ether extraction of phospholipid from the alcohol-ether extract, or by the presence in the alcohol-ether extracts of phosphorus compounds which were not phospholipid and which were insoluble in petroleum ether.

Several experiments were accordingly made to determine whether the degree to which the alcohol-ether extract was dried made any consistent difference in the amounts of phosphorus in the petroleum ether extract. The results showed that no great differences could be attributed to this factor. Moist diethyl ether was found to behave like petroleum ether as a solvent.

It was felt that a working hypothesis was afforded by the idea that a considerable portion of the phospholipid was the more insoluble sphingomyelin which was extracted by alcohol and ether from the plasma, but only partially dissolved by the petroleum ether.

In order to test this hypothesis 250 cc. of cow plasma were extracted with 2.5 liters of alcohol-ether mixture. The extract was concentrated *in vacuo* to a volume of 100 cc., the temperature being kept below 40°. The concentrated extract was placed in a

continuous extractor and extracted with petroleum ether at room temperature for 10 hours. The petroleum ether extract contained 11.4 mg. of phosphorus. The water layer was poured into an excess of acetone. The slight precipitate formed was centrifuged off. The precipitate contained 0.2 mg. of phosphorus, while the acetone fraction contained 0.3 mg. of phosphorus. Thus the total phosphorus in the alcohol-ether extract was the sum of 11.4, 0.2, and 0.3 mg. or 11.9 mg. The 0.5 mg. of phosphorus not dissolved by the petroleum ether represents about 4.2 per cent of the total phosphorus in the alcohol-ether extract.

95 cc. of the above petroleum ether extract, representing 237.5 cc. of plasma, were poured into 500 cc. of acetone. A few crystals of sodium chloride were added to facilitate precipitation of the phospholipids. After settling in the ice box overnight the precipitate was centrifuged down and washed once with acetone. Redistilled ether was added to the precipitate. A slight residue was insoluble. This was centrifuged down, the ether decanted, and 3 cc. of pyridine added to the residue. After heating to 60°, the pyridine solution was centrifuged hot and the supernatant liquid was decanted and set in the cold room. After several days spherocrystals were found in the material which crystallized out of the pyridine. These were examined between crossed nicols in the polarizing microscope. There were a few large, easily distinguishable crystals appearing as a black cross on a white circular background. Nearly all of the material crystallizing from the pyridine appeared to be spherocrystals. From the work of Rosenheim and Tebb (3) it is highly probable that these were composed of sphingomyelin with cerebrosides as impurities.

The ether solution of the acetone precipitate contained 3.62 mg. of phosphorus, which indicated an incomplete precipitation. Portions of the ether were diluted and aliquots used for the determination of phospholipids by Bloor's oxidative method. Six analyses gave an average value of 74.3 mg. A portion of the ether solution was also saponified and the fatty acids extracted with petroleum ether after acidification. Eight analyses by Bloor's oxidative procedure gave an average value of 50.9 mg. for the phospholipid fatty acids. Thus the ratio of phospholipid to phosphorus was 20.5, and the ratio of phospholipid fatty acids to phosphorus was 14.1. These ratios are discussed later.

In the preparation of phospholipid as outlined above, petroleum ether dissolved nearly all of the phospholipid in the alcohol-ether extract, while in the Bloor procedure it did not. The only essential difference in the two procedures is in the method of concentrating the alcohol-ether. When a vacuum is used, petroleum ether serves as a good solvent. When a vacuum is not used, petroleum ether fails to dissolve all of the phospholipids. At the time these observations were being made Lintzel (4) published a method for determining phospholipids in goat plasma in which the alcohol-ether extract was concentrated in a vacuum. We adopted his procedure with certain modifications.
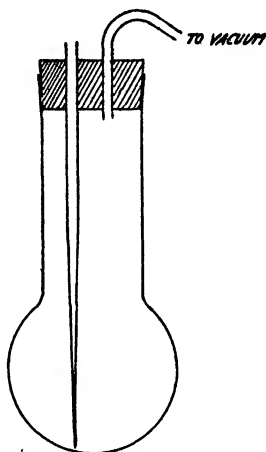
FIG. 1. Apparatus for evaporating alcohol-ether from lipid extract (one-third full size).

Directions for Procedure Adopted

Place 100 cc. of alcohol-ether extract in a round bottom, wide necked flask fitted with a capillary tube, as shown in Fig. 1. Immerse the flask in a water bath kept at a temperature not exceeding 50° and apply a vacuum by means of a water pump. When the contents of the flask are completely dry, add 10 cc. of petroleum ether (redistilled from concentrated sulfuric acid between 35–60°). In adding the petroleum ether wash down the sides of the flask and the capillary tube and blow in the last portion forcibly to break up the mass of material in the bottom of the

flask.   Boil off all but 2 or 3 cc. of the petroleum ether on a steam
bath.   Then add 10 cc. of redistilled acetone (dried over anhy-
drous sodium sulfate) and 3 drops of 4 per cent magnesium chloride
in 95 per cent alcohol.   Allow to stand in the cold room for at least
2 hours and decant the acetone into a 50 cc. centrifuge tube.
Blow into the flask three portions of acetone, about 2 cc. each,
decanting the acetone each time into the centrifuge tube.   Re-
move the acetone by centrifugation.   Heat the flask gently on the
steam bath and with a slow stream of carbon dioxide gas remove
all traces of acetone from the flask.   Then dissolve the precipitate
in chloroform, pouring from the flask into the centrifuge tube.
25 cc. of chloroform make a convenient amount.   A suitable
aliquot may be taken for a phosphorus analysis, in which case it is
unnecessary to centrifuge.   The phospholipids are determined by
Bloor's oxidative procedure after centrifugation of the chloroform
solution.   To determine the phospholipid fatty acids evaporate
the chloroform solution to dryness in a 125 cc. Erlenmeyer flask
on a steam bath.   Dissolve the residue in 10 cc. of redistilled 95
per cent alcohol and add 0.15 cc. of 50 per cent potassium hydrox-
ide.   After heating on the stream bath to dryness, with the
rate of heating adjusted so that 20 to 30 minutes are required,
add 2 cc. of sulfuric acid (1:3) and extract with petroleum ether
(b. p. 35–60°).   Bloor's procedure is then used for the determination
of the fatty acids.

This method differs from the procedure used by Lintzel, in that,
instead of centrifuging down the precipitate of phospholipids, he
used a filter and instead of chloroform he used moist ether.   We
found that either solvent gave the same results, but that chloro-
form required less time for complete solution of the phospholipids
and gave more consistent results.   Lintzel, using goat blood
plasma, found that 1 part of phosphorus in the moist ether solu-
tion of the phospholipids was equivalent to 16.5 to 16.8 parts of
fatty acid.   He determined the phosphorus in the moist ether
solution and used the theoretical equivalent of 18 in calculating
phospholipid fatty acids from the phosphorus analysis.

Results

By the method outlined above on thirty-nine plasma samples
the average phosphorus content in the alcohol-ether extract was

equivalent to 8.44 mg. per cent and in the chloroform it was 8.09 mg. per cent. The average ratio of phospholipid fatty acids to phosphorus in the chloroform solution was 14. The average ratio of phospholipids to phosphorus, based on twelve determinations, was 28. In the case of nine cell samples the average phosphorus content in the alcohol-ether was 16.34 mg. per cent and in the chloroform solution, 14.24 mg. per cent. The average ratio of phospholipid fatty acids to phosphorus in the chloroform solution was 15.2. Some typical data obtained on plasma, which serve to

TABLE I

Some Typical Results Obtained by Modified Extraction Procedure

Cow No.	Source of blood	Phosphorus in alcohol-ether	Phosphorus in chloroform	Phospholipid fatty acids	Phospholipid	Ratio of phospholipid to phosphorus	Ratio of phospholipid fatty acid to phosphorus
		mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*		
J-16 (dry)	Artery*	9.0	8.2	107			13.1
	Mammary vein	9.0	8.6	99			11.5
	Jugular "	9.1	8.8	115			13.1
T-20 (lactating)	Artery	6.6	6.3	106	176	27.9	16.8
	Mammary vein	6.5	6.2	94	166	26.8	15.2
	Jugular "	6.9	6.4	104			16.3
P-24 (lactating)	Artery	10.6	10.5	155	291	27.7	14.8
	Mammary vein	10.5	10.2	146	291	28.5	14.3
	Jugular "	10.7	10.0	138			13.8
R-25 (lactating)	Artery	8.0	8.0	124	231	28.9	15.5
	Mammary vein	8.3	8.0	126	245	30.6	15.7
	Jugular "	8.3	8.1	134			16.5

* The arterial blood was drawn from an artery in the wall of the vagina.

illustrate the variability of the results, are presented in Table I. The three blood sources shown were used as a part of our study, to be reported elsewhere, confirming the conclusion of Blackwood, and also of Lintzel, that phospholipid is not the blood precursor of milk fat.

DISCUSSION

Bloor (2) found that the lipoid phosphorus method and the oxidative procedure gave comparable results with dog and human

plasma. A satisfactory explanation for the failure by his procedure to isolate all of the phospholipid from cow plasma extract cannot be given at this time. It is interesting to note that, if an extraction with petroleum ether is made after drying *in vacuo*, a considerably greater amount of phospholipid is extracted than when drying is done under atmospheric pressure. In the few instances tried, however, less phospholipid was obtained by extraction after drying *in vacuo* than was obtained by our procedure previously outlined. It is also interesting to note that, when about two-thirds of the alcohol-ether mixture was boiled off without a vacuum and then dried down completely *in vacuo*, an extraction with petroleum ether gave results no different from those obtained when no vacuum was used. This indicates that temperature, and not the physical state due to the method of drying, is responsible for the relative insolubility of phospholipids in petroleum ether when Bloor's procedure is used. Recently Kirk, Page, and Van Slyke (5) have modified the Bloor isolation procedure by evaporating at lower temperature, and Man (6) has recommended the use of a vacuum and the exclusion of oxygen.

No explanation can be made for the low ratio of 20.5 between the phospholipids and phosphorus found for the ether solution of the phospholipids obtained from the 250 cc. of plasma. The high ratio of 28 found in the application of the analytical procedure adopted might be due to contamination with oxidizable compounds, such as the so called "carnithine" substance (7). The low ratio of approximately 14 found for phospholipid fatty acids to phosphorus can be partially or perhaps wholly accounted for by the finding of sphingomyelin in plasma. Sphingomyelin contains but one fatty acid residue for each atom of phosphorus and Levene (8) found that drastic treatment was necessary to split off this fatty acid. Under the rather mild saponification treatment employed in our analytical procedure probably none of the fatty acids from sphingomyelin would be included in the determination. After this work was completed, Thannhauser and Setz (9) published a method for the quantitative determination of diaminophosphatide in serum. Using serum from normal adults, they found the ratio of diaminophosphatide to total phosphatide to be between 1:2 and 1:3. If it be assumed that the lowering of the ratio is due entirely to the presence of sphingomyelins, and that

the fatty acid is not split off in the saponification, it can be calculated that, when the ratio is 14, about 22 per cent of the phospholipids present are sphingomyelins.

Stewart and Hendry (10) made a study of the phosphorus distribution in the various fractions obtained with Bloor's procedure, using whole blood from an unspecified source. They obtained a recovery of about 97.5 per cent of the alcohol-ether-soluble phosphorus in the moist ether solution of the isolated phospholipids. They determined the fatty acids obtained by saponification and found a ratio of fatty acid to phosphorus of about 13:1 and concluded that the phospholipid of whole blood must consist of approximately 50 per cent sphingomyelin and 50 per cent of a mixture of lecithins and cephalins.

Bloor (11) and Schaible (12), working with fairly large amounts of plasma, obtained low yields of phospholipid fatty acids and suggested that decomposition occurred during the process of isolation. This factor may play a rôle in the present case. It may be noted, however, that alcohol-ether extracts of plasma when stored for several months gave ratios no lower than extracts used a few days after preparation. It was also found that identical results for lipoid phosphorus, phospholipid, and phospholipid fatty acids were obtained on a plasma sample, one portion of which was used the same day it was drawn from the cow and the other portion of which was stored for nearly a month at 7° and then 2 days at 27° before being used.

Since the ratio of phospholipid fatty acids to phosphorus is low for cells as well as for plasma, indirect evidence is furnished that sphingomyelin is present in the cells of cow blood.

SUMMARY

A suitable procedure has been found for the determination of the phospholipids and phospholipid fatty acids in the plasma and in the cells of bovine blood, which gives results in substantial agreement with those obtained by the lipoid phosphorus method.

Direct evidence has been obtained that sphingomyelins are present in the phospholipids of plasma, and indirect evidence that this phospholipid is present also in cells.

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THE APPLICATION OF SPECTROGRAPHIC ANALYSIS TO THE QUANTITATIVE DETERMINATION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM IN BIOLOGICAL FLUIDS

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Qualitative analysis with the spectrograph has occupied an important place in biological chemistry for many years, as is evidenced by the large number of papers appearing in biological journals in which the spectrograph has been used to prove the presence of traces of various elements. Quantitative spectrographic analysis, on the other hand, has been largely neglected, although the speed of the method and the simplicity of the technique involved should make it very useful. This neglect appears to be due to a general impression that exact analyses cannot be made with the spectrograph. The purpose of the work described in the present paper was to show that quantitative spectrographic analyses of biological material could be carried out with an accuracy comparable to that obtained by chemical analysis. Since this is true for elements which occur in higher concentrations, such as those mentioned in the title, the spectrograph should have a decided advantage for elements occurring in lower concentrations.

The method of analysis which has been used is similar to one recently described by Duffendack, Wiley, and Owens (1), but involves certain improvements, especially in the source used for the excitation of the spectra. In developing this source, the primary consideration has been the accuracy of the results. An effort has also been made to develop a source which will remain permanently in adjustment, and will not be disturbed by such operations as loading, and cleaning after use.

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Source

The source which was finally adopted, as best meeting these requirements, consists of a spark between two horizontal jets from which the solution drips. A preliminary description of this source was presented to the American Society for Testing Materials, at the 1936 annual meeting (2). Two forms of this apparatus have been used, which differ only in the manner in which the
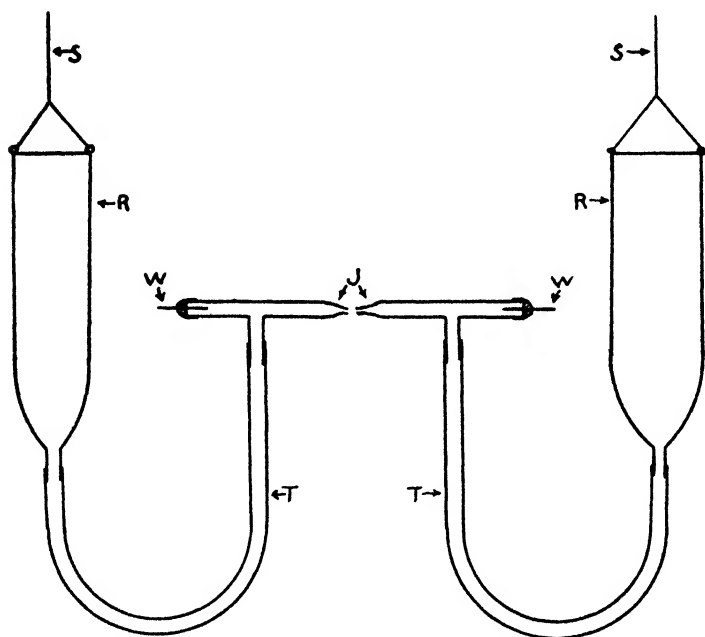
Fig. 1 First form of source   *J* indicates the jets; *W* leads, gold wires through rubber caps; *T* rubber connecting tubes; *R* reservoirs; *S* strings leading to winding drum.

flow of solution through the jets is produced and controlled. In the first form, illustrated in Fig. 1, two reservoirs are connected to the jets by means of rubber tubing. The reservoirs are straight glass tubes of uniform cross-section and are suspended on strings which pass over a drum rotated by a motor at constant speed. As the reservoirs are raised, the solution spills out through the jets, keeping the level of the liquid in the reservoirs at the height

of the jets.   The rate of flow can be regulated by changing the diameter of the reservoirs or the size of the drum.

In the second form, the reservoirs are rigidly attached to the jets by glass or quartz tubing, and are bent along the arc of a circle, as shown in Fig. 2.   The center of the circle lies on the axis of the jets, and the whole apparatus is rotated about this axis with uniform angular velocity.   Thus the spark between the jets remains fixed in position while the liquid is spilled out through them by the rotation.   This form has an advantage over the first, in that it requires less solution for an analysis, due to the elimination of most of the connecting tubing.

The jets may be made either from glass or quartz, the latter material being preferable because it is not damaged when the
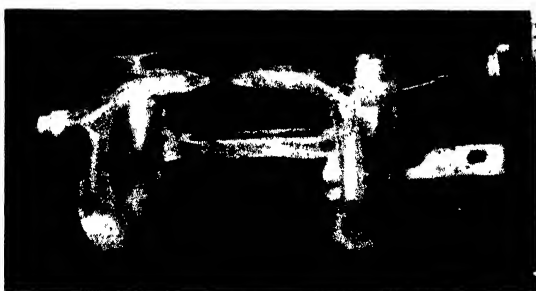
Fig. 2. Photograph of rotating jet form of source

spark strikes to the wall of the jet, as may happen if a bubble passes through.   The jets are rigidly attached to each other by a rod of the same material which serves to keep them at the proper distance from one another.   They are made from tubing with an internal diameter of about 5 mm., which is large enough to prevent undue heating of the solution by the passage of the current through it, and are drawn down at the end to a diameter of about 0.75 mm. Electrical contact is made with the solution by means of gold wires passing through rubber plugs or caps.   The source is cleaned after use simply by flushing with distilled water.

A source, somewhat similar in form, consisting of a spark between two electrodes of the solution to be analyzed has also been used by Lukas (3), who obtained rather erratic results with it. We have found that when precautions are taken to keep the jet

separation and the rate of flow constant, this source gives very consistent results.

The spark is excited by a 1 kilovolt-ampere, 25,000 volt transformer, run on 110 volt, 60 cycle alternating current and regulated by a rheostat in the primary circuit. No condensers or extra inductances are used in the circuit, which is highly inductive, due to the self-inductance of the transformer used. The spark obtained in this way is very arc-like in character, and excites only the lowest members of the arc series in the spectra of the elements. Consequently, there is practically no choice in the lines which may be used in the analyses. The lines used in the present work were as follows: magnesium 2852 Å., sodium 3303 Å. (unresolved pair), potassium 4044 Å., calcium 4226 Å. In addition to these elements, copper and indium were introduced into the solutions in known amounts to serve as internal standards, and the copper lines at 3247 Å. and 3274 Å. and the indium line at 4101 Å. were used. The use of copper as an internal standard for biological fluids might be questioned on the ground that some copper may be present normally in these materials, but tests indicated that the amount to be expected is so small compared to the amount added that no appreciable error is introduced.

A number of experiments were carried out to determine the effects of changes in the set-up. It was found that changes in the spark current and jet diameter had no important effect on the relative intensities of the lines. The spark current was limited by the danger of boiling the solution in the jets, which caused the spark to strike to the walls of the jets. With the dimensions given above, a current of 33 milliamperes was found to be quite safe.

Changes in the rate of flow of solution and the separation of the jets had a very marked effect. The spark became unstable when a gap of more than about 5.5 mm. was used, and the calcium line became very weak when short gaps were used. The most satisfactory gap between the jets was found to be 4.5 to 5 mm. The calcium and potassium lines were very sensitive to changes in the rate of flow of the solution and showed changes in opposite directions. If the rate of flow was increased, the calcium line became stronger, and the potassium line weaker. At the same time, the results of the analyses for calcium became more consistent, while the results for potassium became somewhat erratic. The effects

of this change are shown in Table I. The rate of flow will thus be regulated according to whether accuracy is more important for calcium or for potassium. A rate of flow of about 1 cc. per minute from each jet was found to give the most satisfactory results for

TABLE I

Analyses of Test Solutions Obtained with Flow of 1 Cc. or 4 Cc. per Minute from Each Jet

All results are in mg. per 100 cc.

Magnesium		Sodium		Potassium		Calcium	
Found	Theoretical	Found	Theoretical	Found	Theoretical	Found	Theoretical
Flow of 1 cc. per min.							
31.3	30.0	·247	250	195	200	7.5	10.0
6.3	7.5	180	175	175	175	8.5	9.0
9.4	10.0	312	310	202	210	7.3	9.5
11.2	10.0	314	300	194	200	22.1	20.0
10.5	10.0	241	250	168	175	14.2	15.0
10.7	10.0	255	250	170	175	15.1	15.0
6.9	7.0	335	350	247	250	10.4	8.0
7.3	9.0	300	300	151	150	12.0	11.0
9.3	9.0	299	300	151	150	11.4	11.0
10.5	10.0	295	300	207	200	23.0	20.0
Flow of 4 cc. per min.							
10.8	10.9	285	300	160	160	19.1	19.9
8.7	8.9	372	373	209	190	14.5	14.3
9.7	8.9	362	373	208	190	14.7	14.3
14.1	12.8	301	300	182	192	24.6	23.5
12.3	12.0	307	300	207	222	19.0	18.8
10.3	10.0	297	300	191	200	20.0	20.0
11.4	10.0	296	300	186	200	20.3	20.0
8.6	9.0	284	300	129	150	10.1	11.0
9.7	10.0	246	250	169	175	14.6	15.0
6.3	7.1	326	319	205	200	10.0	10.1

potassium, while a flow of 4 cc. per minute gave better results for calcium.

Preparation of Solutions

Preparation of Standard Solutions for Working Curves and for Tests—In order to obtain a working curve for an element, a series

of solutions was made up containing varying concentrations of that element. In each solution of the series a fixed amount of each of the other elements to be determined was included. These amounts were chosen to be approximately representative of normal urine, so that when analyses were made from the working curves, the process of correcting for the effect of one element on another, described by Duffendack, Wiley, and Owens (1), was greatly simplified. It was found sufficient, in using these curves, to make a single approximation from the correction curves.

Standardized stock solutions of the chlorides of the four elements and of the internal standards were used in making up these standard solutions. To increase the conductivity of the solutions, and to reduce the effect, observed by Duffendack, Wiley, and Owens (1), of variable chloride and sulfate concentrations on the relative intensities of the lines, hydrochloric and sulfuric acids were added in such quantities that acidity, total chloride, and excess sulfate were the same for each solution. By excess sulfate is meant the amount of sulfate above that required to combine with the metal bases present. In each solution the total chloride and excess sulfate each amounted to 0.05 equivalent per 100 cc. of solution. Unknown test solutions were made up from the same stock solutions and had the same acid concentrations as the standard solutions.

Preparation of Urine Samples—Duffendack, Wiley, and Owens (1) have recommended the following procedure for the preparation of urine for analysis: "Remove the phosphates from the urine, ash the filtrate with sulfuric acid until the excess acid is removed, dissolve the ash with 10 cc. of 5 N hydrochloric acid or its equivalent, add to this solution 5 cc. of 10 N sulfuric acid and [the internal standard] and dilute to 100 cc. [the original volume]." This procedure was tested in the present work, by comparison with chemical analyses, and it was found that there was invariably an apparent decrease in potassium and a roughly equivalent increase in sodium. The difference in potassium analyses was small (5 to 8 per cent of the amount present), but well above the normal errors of the method. To avoid this difficulty a less drastic method of oxidation was adopted. The urine was heated first with nitric acid, then with a mixture of nitric and sulfuric acids until most of the nitric acid was driven off. Obviously, such a method made it

impossible to duplicate exactly the chloride and sulfate concentrations of the standard solutions and introduced a small unknown amount of nitric acid, but tests indicated that the variations introduced into the analyses by this procedure were too small to be detected. The following is the detailed method of preparation finally adopted: A sample of urine—usually 300 to 400 cc.— was pipetted into a 500 cc. volumetric flask, and made just acid to methyl red with 5 per cent acetic acid, to prevent precipitation of tricalcium phosphate. The flask was then placed in a water bath and a 3.5 per cent solution of uranyl acetate was added slowly until the precipitation of phosphate was complete, a 10 per cent solution of potassium ferrocyanide serving as an outside indicator. The mixture was diluted to volume, shaken, allowed to stand for an hour, and filtered. An aliquot of the filtrate, 100 to 150 cc., was pipetted into a 300 cc. Kjeldahl flask, 25 cc. of concentrated nitric acid were added, and the mixture was boiled down to a small volume. Then 5 cc. of 10 N sulfuric acid were added and the mixture was heated carefully until most of the nitric acid was expelled. The contents of the flask were then transferred to a 100 cc. volumetric flask, and 10 cc. of 5 N hydrochloric acid and the proper quantities of internal standard were added. Upon diluting to volume, the specimen was ready for spectrographic analysis.

The volume of material used above is, of course, much greater than is necessary, as the whole operation can be carried out on a much smaller scale. Complete determinations have been carried out in cases where the amount of urine available was as small as 10 cc.

Method of Analysis

The method of analysis used is essentially that described by Duffendack, Wolfe, and Smith (4), although it differs in some details. A working curve is constructed for each element by plotting the logarithm of its concentration against the logarithm of the intensity of its line relative to a standard line. When the logarithms are used, the resulting curve is a straight line over most of its length. In addition to these curves, another set is drawn, showing the effect of each element on the intensities of the lines of the other elements, and corrections are made to the

analyses from these curves. Examples of these curves have been given in a previous publication (2). The line intensities were measured by the method of Thomson and Duffendack (5).

Results of Analyses

Table I gives the results of analyses of a number of test solutions. Each of the analyses given is the result of a single set of measurements on one plate, and these results were obtained before the

TABLE II

Comparison of Spectrographic and Chemical Analyses of Urine Samples with Flow of 1 Cc. per Minute through Each Jet

The concentrations are expressed in mg. per 100 cc.

Magnesium		Sodium		Potassium		Calcium	
Spectro-graphic	Chemical	Spectro-graphic	Chemical	Spectro-graphic	Chemical	Spectro-graphic	Chemical
10.0	11.8	361	346	243	245	14.2	13.9
10.0		355		234		13.1	
11.0		351		237		13.6	
10.6		362		240		14.5	
7.0	7.5	322	335	213	210	10.9	10.6
7.4		342		205		10.9	
6.9		343		209		11.7	
7.3		332		218		11.4	
6.8	7.5	351	335	211	210	12.5	10.6
7.2		359		214		13.2	
6.9		343		212		12.1	
7.1		347		215		12.0	
11.0	9.9	390	391	195	204	12.3	11.3
10.1		384		188		12.1	
9.6		387		197		12.7	
9.6		385		182		13.7	

theoretical values were known to the analyst. A large number of such analyses were carried out in testing the method, but only a number of representative examples are given in Table I.

Table II shows a comparison of chemical and spectrographic analyses of urine samples. Large samples were made up, and a number of spectrographic analyses were made on each sample. The methods used for the chemical analyses were as follows: magnesium, Briggs' method (6); sodium, method of Butler and

Tuthill (7); potassium, method of Shohl and Bennett (8); calcium, method of Shohl and Pedley (9).

The method has also been applied to analyses of saliva. Owing to the lower concentration of the metal salts in saliva, it was necessary to reduce the volume of the saliva to one-fifth its original volume before analysis, but otherwise the treatment was exactly the same as for urine. Three analyses of one saliva sample gave the following concentrations: magnesium 0.16, 0.15, 0.15 mg. per 100 cc., sodium 36, 36, 36 mg. per 100 cc., potassium 89, 88, 87 mg. per 100 cc., calcium 5.3, 5.2, 5.6 mg. per 100 cc. No chemical analyses were made of this sample.

Practical use has been made of the method in connection with certain metabolism experiments to be reported by Dr. R. L. Grant from the laboratory of Dr. L. H. Newburgh at the University Hospital in Ann Arbor, and it was found to give very satisfactory results.

DISCUSSION

The results given in Tables I and II show that the method here outlined will give fairly accurate analyses for sodium, potassium, and, under proper conditions, for calcium. Slightly less accurate analyses can also be made for magnesium. The ranges of concentrations over which the method can be used without modification are summarized in Table III. These ranges can undoubtedly be extended in a number of cases by slight modifications, such as the use of other lines for the analyses, if such extensions are necessary. The ranges have been divided into two parts, possible and favorable. The limits are not very definite, and depend to a considerable extent on the conditions under which the source is used. The lower limit of the possible range is the concentration at which the line used just appears on the plate, under normal conditions of excitation and exposure. Since such weak lines cannot be measured accurately, the lower limit of the favorable range has been placed a little higher, where the line has become strong enough for accurate measurement. The upper limits are set by a variety of considerations. For calcium, the upper limit is determined by the solubility of calcium sulfate. There appears to be some danger of precipitation above the upper favorable limit given. The upper possible limit for sodium and potassium is set at the

concentration at which the spark becomes unstable. The other upper limits are set by considerations of line intensities.

Within the favorable range, the results obtained for sodium and potassium show variations of the same order of magnitude as those obtained by running the same plate through the microphotometer several times. In other words, the fluctuations in the source are no greater than the errors in reading the microphotometer. In the course of the tests made on the source, 97 test solutions were analyzed for sodium. Of these analyses, twenty-five were within 1 per cent of the theoretical value, 82 were within 5 per cent, and only three were more than 7 per cent off. The largest error found was less than 9 per cent of the amount

TABLE III

Concentration Ranges over Which Analyses Can Be Carried Out

The concentrations are expressed in mg. per 100 cc.

Element	Possible range		Favorable range	
	Low	High	Low	High
Magnesium...........	0.1	100	1.0	10
Sodium..............	10	1000	50	700
Potassium...........	50	1000	100	800
Calcium.............	5	Indefinite*	10	100

* The limit for calcium is set by the solubility of calcium sulfate in the acid solution which is used for the analyses.

of material present. The analyses for potassium are slightly better, in general, than those for sodium, because the intensity of the potassium line increases a little more rapidly with concentration than the sodium line, making errors in reading the microphotometer less important. The intensity of the magnesium line, on the other hand, increases more slowly with concentration, so that errors in reading the microphotometer are about twice as important for magnesium as they are for potassium. Thus larger errors are to be expected in the magnesium analyses, but this factor is not sufficient to account for the observed variations. It appears that there are uncontrolled fluctuations of the magnesium line intensity with this source which make the results somewhat erratic for magnesium.

SUMMARY

A quantitative method of spectrographic analysis for biological fluids has been presented, which has proved satisfactory both in laboratory tests and in practical use. The method has some advantage over chemical methods in speed, as, after the solutions are prepared, one man can easily carry out the analysis of twenty solutions for all four metals in a day. Another advantage of the method is its complete freedom from any operations which require judgment on the part of the operator. After the solutions are prepared, any errors which occur will be apparatus errors, and we have found that an intelligent laboratory technician gets the same accuracy with the method as the men who developed it. The average error in a large number of analyses for sodium and potassium was less than 3 per cent of the amount present, and the maximum error was less than 10 per cent of the amount present. Accuracy is slightly less for analyses of calcium and magnesium.

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THE REACTIONS OF AMINO AND IMINO ACIDS WITH FORMALDEHYDE

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A basis for the systematization of the equilibria between formaldehyde and amino acids has been developed and utilized in a study of the formol titration (9–11). The present paper contains data for several more amino and imino acids, a study of the slow reaction of asparagine with formaldehyde, and discussion of certain points in the literature.

Equilibria of Amino and Imino Acids in Formaldehyde

The experiments consist of the determination of the variation of the apparent dissociation constants of the amino or imino groups with the formaldehyde concentration. A solution of the substance with the necessary amount of NaOH to half neutralize the nitrogen group was prepared (pH is equal to pK_n) and titrated with a concentrated solution of formaldehyde (9, 11). The pH values, which are equal to pG_f, were measured with the H_2 electrode. They are plotted in Figs. 1 and 2 against the logarithms of the formaldehyde concentrations. The substances reported on are *dl*-alanine, *dl*-valine, *l*-aspartic acid, *l*-tryptophane, *dl*-sarcosine, and *l*-hydroxyproline. The purity of each was checked by the Kjeldahl nitrogen, the Van Slyke amino nitrogen, and the acid and alkali equivalents.

Constants for Equilibria—The data of Figs. 1 and 2 were analyzed as previously described (9) for the determination of L_1, the association constant of the anion for 1 mole of formaldehyde, and L_2, the association constant of the anion for 2 moles of formaldehyde. The curves of Figs. 1 and 2 were plotted from these two constants and the appropriate pK_n by the use of the equation

$$(1) \qquad pG_f = pK_n - \log (1 + L_1F + L_2F^2)$$

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in which F is the formaldehyde concentration in moles per liter. The values of L_1 and L_2 are given in Table I, along with those previously available. Attention is called to the fact that L_2 is zero for all of the imino acids and for tryptophane. This means that the imino acids react with only 1 mole of formaldehyde
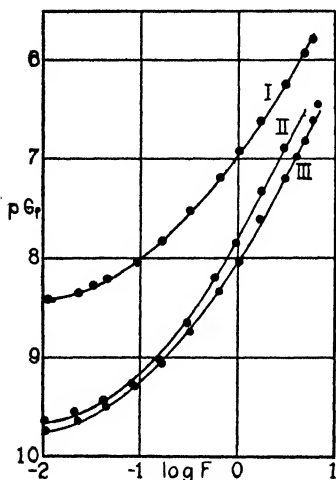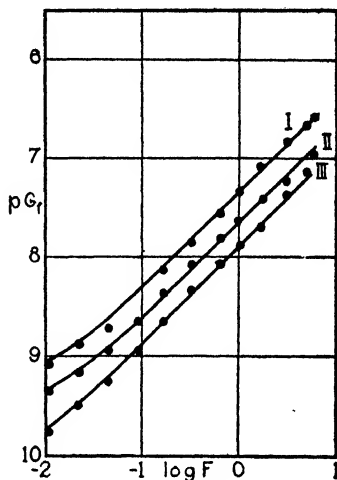
FIG. 1.   FIG. 2.

FIG. 1. The ordinate scale represents the apparent dissociation constants of the amino acids; the abscissa, the logarithms of the formaldehyde concentrations in moles per liter. Curve I, valine (1 has been subtracted from the pG_f values for plotting); Curve II, dl-alanine; Curve III, aspartic acid.

FIG. 2. Ordinate and abscissa as in Fig. 1. Curve I, tryptophane; Curve II, sarcosine; Curve III, hydroxyproline.

under our conditions. The constants for the approximation equations

(2) $pG_f = pK_nL_2 - 2 \log F$

for the imino acids except tryptophane and

(3) $pG_f = pK_nL_1 - \log F$

for the imino acids and tryptophane have been read from Figs. 1 and 2. These constants are expected to differ slightly from the

calculated values (*i.e.* $pK_n - \log L_2$) because of the approximate nature of Equations 2 and 3. The values of the constants are given in Table I.

TABLE I

Formol Constants of Amino and Imino Acids at 30°

Amino acid	pK$_2$	L_1*	L_2*	pKL$_2$†	9% CH$_2$O	
					pG$_f$	pG$_f$‡
Glycine..........................	9.60	60	290	6.65	5.70	5.92
dl-Alanine........................	9.72	22	75	7.81	6.86	6.96
l-Leucine.........................	9.50	16	35	7.87	6.92	
dl-Valine.........................	9.50	22	12	8.20	7.25	7.47
dl-α-Aminophenylacetic acid§........	8.84	13	77	6.90	5.95	
l-Phenylalanine....................	8.99	16	23	7.57	6.62	6.80
l-Tyrosine........................	9.07	10	5	8.45	7.50	
				pKL$_1$		
l-Tryptophane.....................	9.27	83		7.35	6.88	
l-Proline.........................	10.30	112		8.25	7.78	
l-Hydroxyproline..................	9.56	79		7.66	7.19	
dl-Sarcosine......................	10.06	151		7.88	7.41	
				pKL$_2$		
d-Lysine,‖ pK$_3$...................	10.56	240	309	8.10	7.15	
d-Arginine,‖ pK$_2$.................	8.91		6×10^5	3.50	3.40	
l-Histidine, pK$_2$.................	9.17				7.90	
d-Glutamic acid pK$_2$..............	9.32	22	24	7.87	6.91	
l-Aspartic " "	9.83	26	37	8.16	7.21	

* L_1 is the association constant of the amino or imino acid anion for 1 molecule of formaldehyde (called K_2 in a previous paper (9)). L_2 is the association constant of the amino acid anion for 2 molecules of formaldehyde (called K_3 in a previous paper (9)).

† This constant will differ from $pK_n - \log L_2$, because it is derived for an approximation formula and is read directly from the curves.

‡ Data of Dunn and Loshakoff ((4) p. 359).

§ In a previous paper, this substance was erroneously labeled β-phenyl-α-aminoacetic acid.

‖ For a complete description of the behavior of these compounds in formaldehyde see Levy (9).

Methods—The use of the hydrogen electrode in formaldehyde solutions, especially at high concentrations, has been criticized by Tomiyama (17). Balson and Lawson (1) have since published results obtained with this electrode and confirm our experience that it is reliable even in 5 or 6 M formaldehyde. A comparison of our data with the glass electrode measurements of Dunn and Loshakoff ((4) p. 691) and Dunn and Weiner (5) shows that in 9 per cent formaldehyde there is an approximate agreement (the last two columns of Table I). The differences are probably due to the differences in temperature and salt concentrations.

Polymerization of Formaldehyde—It is not probable that the polymerization equilibrium of formaldehyde, which does not involve either liberation or absorption of H ion, is affected by pH. Tomiyama is, however, unwilling to accept data at higher formaldehyde concentrations because of a supposed effect of pH on the polymerization equilibrium. He supports this by reference to a paper by Jahoda (6) in which the conclusion is reached that alkalinity favors depolymerization. The experimental data in Jahoda's paper are drawn from a polarigraphic study of formaldehyde solutions. The effects that led to the conclusion could probably be explained as well on the basis of ionization of formaldehyde in the alkaline medium (10).

The rate of attainment of polymerization equilibrium does depend on pH, as shown by Wadano, Trogus, and Hess (18). Using interferometer readings as a measure of rates, they found a minimum rate between pH 2 and 4 and a rapid rate at pH 6 and above. For this reason and because of the consistency of our data we believe that the polymerization equilibrium had been reached at each point reported. (In the case of arginine the low pH values were perhaps the cause of the slow attainment of equilibrium (11).)

The polymerization equilibrium is such that the free formaldehyde concentration remains nearly proportional to the total concentration over the significant range. Parks and Huffman (12) state that the partial vapor pressure data of Ledbury and Blair (8) on formaldehyde solutions follow Henry's law approximately. The constants L_1 and L_2 for our equilibria may carry a concealed polymerization constant in the same way that the

hydration equilibrium constants of NH_3 and CO_2 are concealed in their commonly used acid-base constants.

Calculation of Constants—The reactions which Tomiyama set up as the basis of his calculations are the hydrogen ion dissociation of the amino or imino group and the reaction of the anion with 1 molecule of formaldehyde. The two reactions correspond to Equations 1 and 2 of the system we have used (9). He tested his equations by the use of data on glycine,[1] alanine, and proline. His restriction of the formaldehyde concentration to 0.2 M or less has been pointed out as the reason for his failure to find more than 1 molecule of formaldehyde reacting per mole of amino acid (Balson and Lawson (1)). There is no disagreement in the case of proline.

It seems to have been Tomiyama's impression that we considered only the reaction with 2 molecules of formaldehyde. Our papers• (9, 11) plainly state, however, that amino acid anions react with 1 as well as with 2 molecules of formaldehyde. His calculation of $K_{e(2)}$ is not a valid test of our hypothesis under his experimental conditions.

Dunn and Weiner have made measurements similar to ours, using the glass electrode. They confirm the adequacy of approximation Equation 2 for the formaldehyde concentrations between 3 and 10 per cent. The numerical values of their constants differ slightly from ours, but the temperature used by them was 22°, whereas we worked at 30°. They did not test the more exact Equation 1, preferring to use an empirical formula for the more exact description of the changes of pK with formaldehyde concentration. We believe that such formulas should be avoided if an equation based on equilibrium reactions can be used.

Our data do not show evidence for reaction with a 3rd molecule of formaldehyde, as proposed by Balson and Lawson (1). We are not certain of the reason for this. Their final formaldehyde concentrations were usually somewhat higher than ours, and, since the third constants are small, we may have missed them.

End-Point and Error—The end-point calculations given by

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[1] The tabulated data given in Tomiyama's paper for glycine are not consistent with the $K_{e(1)}$ values that he gives. Dr. Tomiyama, in a personal communication, states that this is due to an error of the ratio of glycine to glycine anions given in the table but not made in the calculations.

Tomiyama are quite misleading. The limitations which he set on the formaldehyde concentration remove his data far from any practically used formol titration. The result is that he finds it necessary to extrapolate from 0.2 M to 3.3 M (10 per cent) formaldehyde. It is difficult to justify his extrapolation in view of the existence of our own studies which make it unnecessary and which are incompatible with the method of extrapolation. The use of K_w as an index of the behavior of the solvent towards alkali is erroneous because of the acidity of formaldehyde (11).

Dunn and Loshakoff ((4) p. 359) have performed formol titrations of carefully purified amino acids with the glass electrode. They locate the end-point by the method of slopes. This excellent method is independent of the exact evaluation of the pH of the end-point. Its greatest accuracy is obtainable when the slope of the titration curve at the end-point is maximal (Roller (13)). This is the basis on which we calculated the optimal conditions for the formol titration. The error which we calculated for these conditions and a colorimetric end-point determination accurate to 0.1 pH was 0.5 per cent. For the conditions of Dunn and Loshakoff, however, the expected error is one-third as great if the potential readings are accurate to 0.5 millivolt (Roller (13)). The error becomes proportionately smaller as the potential readings become more accurate.

Behavior of Asparagine in Formaldehyde

For many years the methylene amino structure for the products in the formol titration has been accepted. It is found in nearly all text-books dealing with the subject. Our data require that the amino acids react finally with 2 moles of formaldehyde and that imino acids react with 1. We have therefore reexamined the basis of the methylene amino structure.

The structure is based primarily on a compound isolated by Schiff (14) from asparagine and formaldehyde. He dissolved asparagine in warm water and added formaldehyde. A substance precipitated which lost a molecule of formaldehyde on washing or drying. The residue has the composition of a methylene asparagine. A copper salt was also prepared and analyzed.

Later Cherbuliez and Stavritch (2) showed that when this methylene asparagine was submitted to the Hoffman reaction

a pyrimidine resulted instead of the expected product. Oxidation with $KMnO_4$ also produced a pyrimidine derivative. This and our own data can best be interpreted as showing that methylene asparagine is a reduced pyrimidine.

Two types of experiments were performed. The first began as an attempt to make the same sort of measurements on asparagine that we have made on other amino acids. 0.5 equivalent of alkali was added to a solution of asparagine and formaldehyde
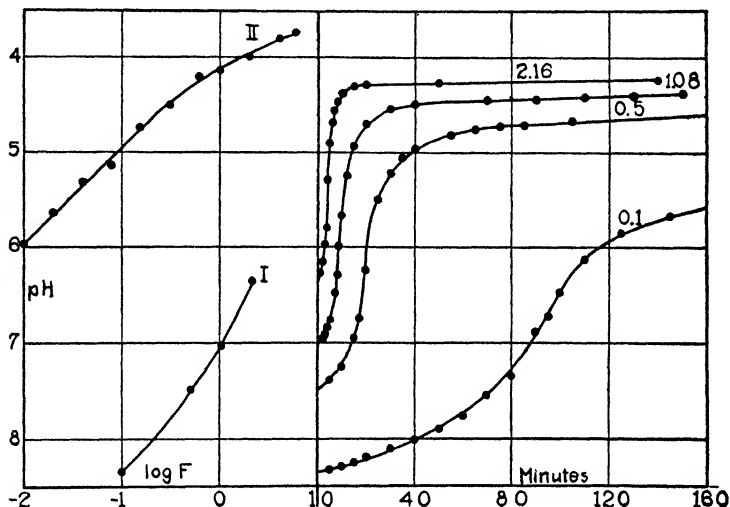
FIG. 3. Reaction of formaldehyde and asparagine. Curve I represents the extrapolated initial points of the time curves. Curve II, the final equilibrium points. The figures above the time curves show the formaldehyde concentrations in moles per liter.

added. The potentials changed in a peculiar manner. The course of the changes is shown in Fig. 3 for four different formaldehyde concentrations and 0.01 M asparagine. Final pH values, also shown in Fig. 3, were measured after 2 or 3 days.

The second group of experiments consisted of determinations of the amino nitrogen by the Van Slyke manometric method after varying periods of contact between the aspargine and formaldehyde. The formaldehyde concentrations, pH, and asparagine concentrations were varied. The formaldehyde concentrations were at least 10 times the asparagine concentrations.

Zero Time-pH—The time-pH curves of Fig. 3 have been extrapolated to zero time and the pH values so obtained plotted against the logarithms of the respective formaldehyde concentrations on Curve I of Fig. 3. The curve so obtained is like that for the equilibria of other amino acids; that is, it reaches a slope of 2 as the formaldehyde concentration increases. It is therefore postulated that there is an instantaneous equilibrium set up which may be formulated like that of other amino acids. The asparagine anions react at once with 1 or 2 molecules of formaldehyde. Our data are not extensive enough to calculate L_1 but pK_2L_2 is 7.05 as obtained by extrapolation along the slope of 2.

Disappearance of Amino Nitrogen—The rate of disappearance of amino nitrogen by the Van Slyke method is consistent with the reaction

$$
\begin{array}{l}
\text{C}{=}\text{O} \\
\phantom{\text{C}}|\text{—NH}_2 \\
\text{CH}_2 \\
\phantom{\text{C}}| \\
\text{CH—NH}_3^+ \\
\phantom{\text{C}}| \\
\text{COO}^-
\end{array}
\;+\; \text{CH}_2\text{O} \;=\;
\begin{array}{ll}
\text{C}{=}\text{O} & \text{NH} \\
\phantom{\text{C}}| & | \\
\text{CH}_2 & \text{CH}_2 \\
\phantom{\text{C}}| & | \\
\text{CH} & \text{NH}_2^+ \\
\phantom{\text{C}}| \\
\text{COO}^-
\end{array}
\;+\; \text{H}_2\text{O}
$$

(4)   (*a*)   (*b*)
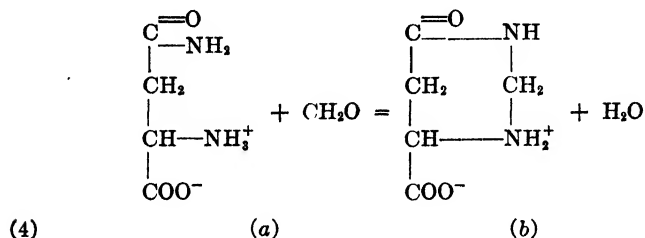
When the formaldehyde concentration is at least 10 times the asparagine concentration, the kinetic equation which fits the reaction is $kF = 1/t \log A/(A - B)$. A is the original concentration of total asparagine. The unimolecular constant kF is proportional to the formaldehyde concentration, as shown in Table II. The fact that the rate is independent of pH or, in other words, of the ionic form of asparagine indicates that the rate of formation of (*b*) is controlled by the rate at which formaldehyde combines with the amide group and not on the rate or extent of its reaction with the amino group. (*b*) is the compound which Schiff (14) isolated and which we think Cherbuliez and Stavritch have shown to be a pyrimidine.

Final pH Values—The pyrimidine formed is an ampholyte with its basic imino group. The final pH values which are given in Fig. 3 show that it is in equilibrium with formaldehyde. The slope of 1 which the curve has indicates that the imino groups

<div align="center">TABLE II</div>

Velocity Constants for Reaction of Asparagine with Formaldehyde at 30°

NaOH	(Asparagine)	CH₂O	kF*	k'

Calculated from disappearance of amino N				
N				
0.005	0.01	0.1	0.0033	0.033
0	0.01	0.1	0.0034	0.034
0	0.01	0.2	0.0057	0.029
0	0.01	0.5	0.0132	0.026
0	0.05	0.5	0.0155	0.031
0.01	0.01	0.2	0.0057	0.029
pH 4.7,	0.01	0.1	0.0033	0.033
acetate	0.01	0.2	0.0059	0.029
	0.01	0.5	0.0143	0.029
pH 6.8,	0 01	0.1	0.0031	0.031
phosphate	0.01	0 5	0.0143	0.028
Calculated from pH-time curves				
0 005	0.01	0.1	0.0028	0.028
0.005	0 01	0.5	0.013	0.026
0.005	0.01	1 08	0.028	0.026
0.005	0.01	2.16	0.60	0.028

* kF and k' values are calculated with logarithms to the base 10.

react with 1 mole of formaldehyde, as do other imino groups. The falling off from a slope of 1 at the higher pH is due to the acceptance of H^+ by the carboxyl group from the imino group, resulting finally in the formation of

$$\char`.=O$$

<pre>
CH₂   CH₂
 | |
CH———NH(CH₂O)
 |
COOH
</pre>
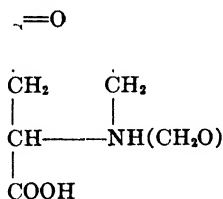
This compound is the one which Schiff isolated and which lost a mole of formaldehyde on washing or drying.

pH-Time Curves—Equation 4 and its accompanying equilibria may be considered for kinetic purposes as the transformation of

an acid at the initial pH and of strength corresponding to pG_{f_1} as read from Curve I to an acid at the final pH and of strength pG_{f_2} read from Curve II of Fig. 3.   The integration of the differential equation derived on this basis leads to the equation

$$kFt = pH - \log{(G_{f_1} + H^+)} - \log{(G_{f_2} - H^+)} - \log{2(G_{f_1} - G_{f_2})}$$

when the logs are to the base 10.   The four values of kF and k calculated by the application of these data to the equation are shown in Table II.   Their agreement with the constants calculated from the amino N method indicates that the same reaction is followed by either method.   Because of the small differences between H^+ and G_{f_1} at the beginning and between H^+ and G_{f_2} at the end of the reaction the calculated values of k are rather erratic except in the central steep part of the curves. It is only for this region that k has been averaged.

Additional Experiments—Methylene asparagine was isolated according to Schiff's (14) directions.   The material was dissolved in water and titrated with acid and with alkali.   The acid branch had a pK of about 2.2 and apparently was homogeneous.   The alkaline branch, however, separated into two parts, one having about pK 6.0 and the other pK 8.8.   The amount of alkali required to reach the break between the two was different according to the time which had elapsed between the preparation of the solution and the titration.   It was evident that the material decomposed partially in solution, probably into asparagine and formaldehyde.   This was confirmed by the amino N which was zero in the fresh solution and increased up to about 70 per cent of the theoretically available if all the compound has decomposed, during the course of several days.   Addition of a small concentration of excess formaldehyde stabilized the compound. Equation 4 is apparently reversible but slow in coming to equilibrium.

The formation of reduced pyrimidine from propylenediamine and formaldehyde has been shown to be probable by Titherly and Branch (16).   The reaction may be a general one for propylenediamines, amides, etc.   Cherbuliez and Stavritch (2) stated the possible significance of the reaction of asparagine with formaldehyde for the mechanism of formation of pyrimidines *in vivo*.

Structure of the Product in Formol Titration

In writing the products in the equilibrium equations set up previously, it was clearly recognized that equilibrium measurements could only serve to eliminate certain structural formulations; they could not establish one. It is evident from the foregoing that the reactions of asparagine with formaldehyde are not analogous to those of the other amino acids. For this and previously mentioned reasons the methylene amino formulation loses its chief support.

Tomiyama (17) has proposed that the diformal compounds result by association of the amino acids with a dimer of formaldehyde, R—NH$_2$—CH$_2$O—CH$_2$O. No differences between amino and imino acids would be expected on this basis, since the 2nd molecule of formaldehyde is attached to the 1st and not to the N.

Since the number of formaldehyde groups which can be introduced corresponds to the number of H atoms attached to N, Balson and Lawson (1) have proposed the following formulations ·as methylolamines

$$\begin{array}{c} R \\ \diagdown \\ \diagup \\ R' \end{array} NH + CH_2O = \begin{array}{c} \diagdown \quad \diagup \\ N \\ | \\ R' \end{array}$$

and if R′ is H
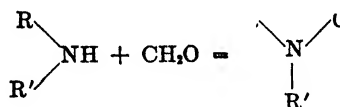
$$\begin{array}{c} R \\ \diagdown \\ N \\ | \end{array} \diagup CH_2OH + CH_2O = R—N \diagup^{CH_2OH}_{\diagdown CH_2OH}$$
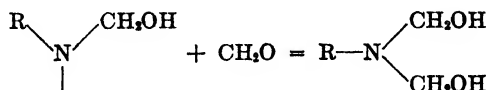
These authors postulated also the introduction of a 3rd molecule of formaldehyde with the formation of a ring. No evidence for the 3rd molecule exists in our data.

These structures are analogous to the accepted structure of acetaldehyde ammonia. They are supported by the formation of tertiary amines on reduction of secondary amines in the presence of aldehydes (15) and the formation of methylated amino acids on heating amino acids and formaldehyde in formic acid (3). The proximity of the O atom to the N would be expected to reduce the affinity of the latter for H$^+$ as it does.

Another possibility is the formation of alkoxyamines. It has been shown that certain alkoxy derivatives of tertiary amines yield aldehydes on decomposition (Jones (7)).

$$(R_3)\!\!\equiv\!\!N\!\!<^{OCH_3}_{OH} \quad = \quad (R_3)\!\!\equiv\!\!N\!\!<^{H}_{OH} \quad + \quad CH_2O$$
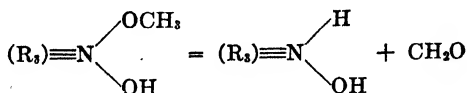
The reverse of this would be made to fit the stoichiometric behavior. Reduction of this type of compound would be expected to yield the original amine and alcohol.

SUMMARY

1. Further equilibrium data of amino and imino acids with formaldehyde are presented.

2. Amino acids may react with 1 or 2 molecules of formaldehyde, whereas imino acids can react with only 1.

3. The reaction of asparagine with formaldehyde results in the formation of a pyrimidine derivative. The rate of this reaction has been measured.

4. Structural formulæ for the products in the formol titration are discussed.

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VITAMIN C IN VEGETABLES

VI. A CRITICAL INVESTIGATION OF THE TILLMANS METHOD FOR THE DETERMINATION OF ASCORBIC ACID*

By G. L. MACK AND D. K. TRESSLER

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During the past few years the Tillmans method for the determination of ascorbic acid has been utilized in a large number of investigations. The method is capable of great precision, but both the original (1) and modified forms (2, 3) may involve large constant errors. While King (4) has shown how interference from other reducing materials in plant tissues can be minimized, a volumetric oxidation method cannot be expected to be highly specific. The problem is further complicated because (a) ascorbic acid may be oxidized to dehydroascorbic acid which is physiologically active but does not react with the titration reagent, (b) while the oxidation of ascorbic acid is reversible, dehydroascorbic acid is unstable and undergoes a further irreversible decomposition (5). Ascorbic acid exists in the tissues of freshly harvested vegetables only in the reduced state (6), but oxidation and further decomposition may occur during the process of extraction.

EXPERIMENTAL

Procedure for Extracting Ascorbic Acid from Vegetable Tissues— If oxidation has been allowed to proceed during extraction, the total amount of ascorbic acid can be recovered for titration by *immediately* reducing the dehydroascorbic acid with hydrogen

sulfide (1, 5). But the necessity of removing excess hydrogen sulfide is a serious objection to the use of this method for routine analysis (7).

The analytical process would be much simpler if, instead of reversing the reaction, the oxidation were prevented throughout the determination. Ascorbic acid is not autoxidizable at pH values below 6.8 (8). Its instability has been ascribed to two catalysts, an oxidase present in practically all vegetables (9) and copper present as an impurity in the reagents (10, 11). The prob-

TABLE I

Relation between pH of Extraction Medium and Amount of Unoxidized Ascorbic Acid Obtained from Vegetables

Extraction medium	String bean		Pea		Carrot	
	pH of extract	Ascorbic acid	pH of extract	Ascorbic acid	pH of extract	Ascorbic acid
		mg. per gm.		*mg. per gm.*		*mg. per gm.*
H_2O	6.2	0.00	6.8	0.00	6.3	0.00
18% $CH_3COOH + 7.5\% K_2C_6H_5O_7$.	4.3	0.05				
3% "			4.0	0 04		
18% " $+ 4\% K_2C_2O_4$.	3.7	0.10				
8% "			3.4	0.17		
18% " $+ 2\% HPO_3$.	2.1	0.15				
8% " $+ 2\%$ "			2.4	0.26	2.3	0.02
8% $CCl_3COOH + 2\% HPO_3$.	1.2	0.19	1.3	0.26	1.2	0.05
5% $H_2SO_4 + 2\% HPO_3$.	0.8	0.20	0.8	0.26	0.8	0.05
10% " $+2\%$ "	0.5	0.20				0.04
15% " $+2\%$ "	0.2	0.20				

lem, then, is one of inactivating or inhibiting the two catalysts of the oxidation reaction.

The catalytic action of copper may be inhibited satisfactorily by adding 2 per cent metaphosphoric acid to the extracting medium (10, 11). The activity of the enzyme is a maximum at a pH value of approximately 5.5 and decreases rapidly as the hydrogen ion concentration is increased. It occurred to us that if the organic acids used in the extraction were replaced by a strongly ionized acid, the pH of the resulting extract would be so low that the enzyme action might be completely inhibited. We investigated the possibility of using sulfuric acid for this purpose, and

compared it with various organic acids which had previously been used. Recently (12) hydrochloric acid has been recommended and should also prove to be quite satisfactory.

Duplicate samples of a number of vegetables were ground with various extraction media by exactly the same technique. The extraction was found to be complete in all cases. In Table I it may be noted that as the acidity of the extract increases the amount of titratable ascorbic acid also increases to a maximum
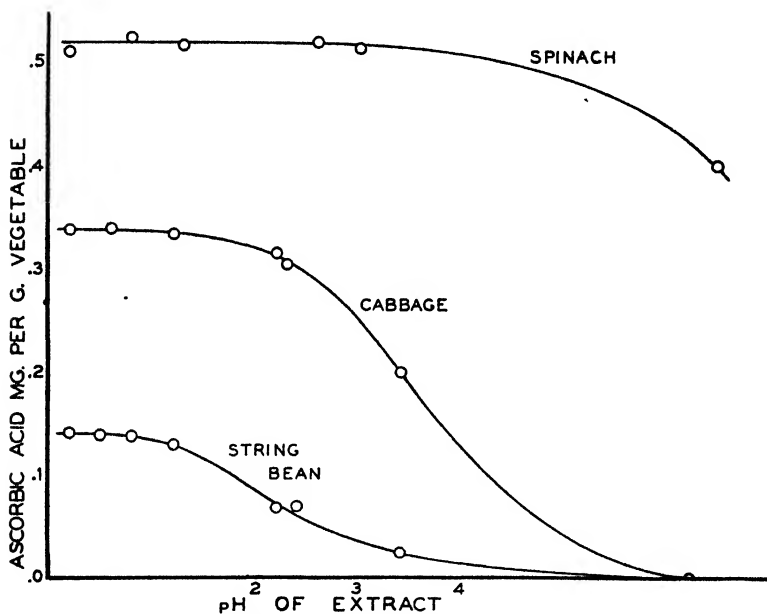
FIG. 1. Effect of pH upon the amounts of ascorbic acid found in vegetable extracts.

value. Further examples of the influence of pH upon the amount of ascorbic acid obtained from different vegetables are shown in Fig. 1.

The maximum amount of ascorbic acid is obtained when the enzyme system catalyzing the oxidation of ascorbic acid is completely inhibited by strong acid. Consequently, the same maximum value is obtained whether dehydroascorbic acid is reduced with hydrogen sulfide or stannous chloride (13) or whether the

oxidation is prevented by inhibiting the reaction with alcohol (6) or with strong acid or heat as shown in Table II.

Examination of Hydrogen Sulfide Treatment for Ascorbic Acid Extracts—The proposed modification of the extraction procedure is not intended to eliminate the hydrogen sulfide treatment in exploratory work.   Indeed, it will now be shown that the use of stronger acid increases the reliability of the results obtained after reduction with hydrogen sulfide.

King (4) has postulated that hydrogen sulfide might reduce substances other than dehydroascorbic acid, which will then re-

TABLE II

Comparison of Effect of Heat and pH upon Activity of Oxidizing Enzymes in Parsnip Extract

Extraction medium	pH of extract	Heating time	Ascorbic acid per gm. parsnip	
			Before reduction	After reduction
		min.	*mg.*	*mg.*
15% H_2SO_4 + 2% HPO_3	0.2	0	0.12	0.11
4% lactic acid	2.7	0	0.08	0.12
Water	6.0	0	0.00	0.13
"	6.0	5	0.13	0.13
"	6.0	7	0.13	0.12
"	6.0	12	0.11	0.13
"	6.0	20	0.12	0.13

act with the titration reagent.   We have discovered an instance in which such interfering substances cause a serious error in the usual ascorbic acid determination.   Freshly sliced carrots were cooked in boiling water for 15 to 17 minutes and drained.   25 cc. portions of the cooking water were mixed with equal volumes of ice-cold 8 per cent acetic, 8 per cent trichloroacetic, and 10 per cent sulfuric acids.   All mixtures contained 2 per cent metaphosphoric acid to inhibit catalytic oxidation by copper.   Duplicate 25 cc. samples were titrated without further treatment. Other samples were saturated with hydrogen sulfide for 10 minutes.   One-half of these were corked and set aside for 20 hours before removing excess hydrogen sulfide.   From the remaining samples excess hydrogen sulfide was removed as quickly as possible with carbon dioxide.

The hydrogen sulfide treatment caused (a) no increase in the titration value of any of the sulfuric acid samples, (b) a small increase in the trichloroacetic acid sample treated 20 hours and in the acetic acid sample treated for 10 minutes, (c) a very great increase of over 80 per cent in the acetic acid sample saturated with H₂S for 20 hours.   To show whether the seeming increases in ascorbic acid were real or only apparent, the differential enzymic method of Tauber, Kleiner, and Mishkind (14) was utilized.

TABLE III

Effect of Hydrogen Sulfide Treatment upon Total Reducing Material in Cooking Water from Carrots Determined in Presence of Various Acids and Expressed As Ascorbic Acid

Time saturated with H₂S	Material titrated	Apparent ascorbic acid		
		5% sulfuric acid	4% tri-chloroacetic acid	4% acetic acid
min.		*mg. per l.*	*mg. per l.*	*mg. per l.*
0	Total reducing material	17	18	18
10	Total reducing material	16	18	21
	Reducing material unoxidized by enzyme	0	0	4
	Ascorbic acid (by difference)	16	18	17
40	Total reducing material	18		20
	Reducing material unoxidized by copper	0		4
	Ascorbic acid (by difference)	18		16
1200	Total reducing material	16	23	33
	Reducing material unoxidized by enzyme	0	5	17
	Ascorbic acid (by difference)	16	18	16

The results given in Table III show definitely that the increased titration value of the cooking water from carrots after hydrogen sulfide treatment cannot be ascribed to reduced ascorbic acid. A similar differential method of analysis with copper as the oxidizing catalyst gave confirmatory results after a short period of saturation with hydrogen sulfide.   This method could not be used when the hydrogen sulfide treatment was prolonged for 20 hours.   A sample containing reducing substances equivalent to 32 mg. of ascorbic acid per liter of cooking water increased in

3 minutes after the addition of 50 parts per million of copper to about 60 mg. per liter, followed by a slow decrease to about 40 mg. per liter. Obviously, such anomalous behavior could not be caused by ascorbic acid. Treatment with mercuric acetate according to the improved procedure of van Eekelen and Emmerie (15) removed the interfering material from the acetic acid extract.

An aqueous extract of Lima beans also was found to give an erroneously high titration value after prolonged treatment with hydrogen sulfide.

In their original work on the hydrogen sulfide treatment, Tillmans, Hirsch, and Jackisch (1) state that the reduction was usually complete in a few minutes but that in certain cases a further slow reaction occurred, so that merely for the sake of uniformity all samples were saturated with hydrogen sulfide for 24 hours. Later workers (15, 16) adopted this procedure without question, but our results, in agreement with those of Bukin (12), indicate that the hydrogen sulfide treatment should only be applied to strongly acidified extracts and for a period of not more than 30 minutes. Cabbage and snap bean extracts treated with hydrogen sulfide for 10, 30, 120, and 1440 minutes showed no significant differences in the amount of ascorbic acid reduced.

State of Ascorbic Acid in Plant Tissues—It has recently (16, 17) been argued that ascorbic acid occurred in a bound or esterified condition in natural foodstuffs, and that it could be liberated by heat or acid. One of us (6) attempted to show by heating duplicate samples of cabbage before and after extraction in an aqueous medium that the apparent increase in ascorbic acid is due to the inactivation of the enzyme. It was conclusively demonstrated that this hypothesis accounted for the greater part of the increase. However, when an aqueous cabbage or pea extract containing no unoxidized ascorbic acid is heated, a small but definite and reproducible amount of reducing material is formed. Further experiments now indicate that this material is not ascorbic acid.

100 mg. of synthetic or natural crystallized ascorbic acid of tested purity were dissolved in 40 cc. of water, acidified with 10 cc. of glacial acetic acid, completely oxidized with 5 gm. of activated charcoal, neutralized with calcium carbonate, and

filtered. The final filtrate contained about 2.5 mg. of dehydroascorbic acid per cc. and did not react with the dye. 2 cc. portions of this solution were mixed with 10 cc. of Sørensen's phosphate buffer mixtures adjusted to pH 6 to 7. Heating these mixtures for 5 minutes in a boiling water bath produced a quantity of reducing material equivalent to 0.29 mg. of ascorbic acid. This is equal to 0.06 mg. of apparent ascorbic acid per mg. of dehydroascorbic acid and is comparable to the value of 0.04 mg. per gm. obtained from both cabbage and pea extracts. These reducing substances were not observed on heating acidified extracts containing dehydroascorbic acid; hence they are probably identical to the physiologically inactive decomposition products of dehydroascorbic acid described by Borsook and coworkers (5).

We wish to express our appreciation to H. Tauber for supplying the purified oxidase used in some of these experiments, and to Z. I. Kertesz for many helpful suggestions during the progress of this work. We are also indebted to F. Fenton, R. B. Dearborn, and S. L. Gould for portions of the experimental data.

SUMMARY

1. The extraction procedure in the Tillmans method for the determination of ascorbic acid has been modified to include the use of an acid which is ionized sufficiently to prevent enzymic oxidation of ascorbic acid.

2. For routine analysis the practicability of preventing the oxidation of ascorbic acid throughout the determination is demonstrated. The consequent elimination of the hydrogen sulfide treatment greatly simplifies the analytical procedure.

3. Substances other than dehydroascorbic acid were reduced by prolonged hydrogen sulfide treatment in weak acid solutions. The use of a strongly ionized acid prevented the interfering material from reacting with the titration reagent.

4. The apparent increase in ascorbic acid on heating an aqueous cabbage extract is caused by the decomposition of dehydroascorbic acid.

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THE FORMATION OF METHEMOGLOBIN BY VARIOUS TISSUES

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In a previous paper (1) it was suggested that the conversion of hemoglobin to methemoglobin by various tissues might be used as a test for the presence of hydrogen peroxide or other peroxides. By this method it was shown that the oxidation of the non-natural isomers of amino acids was accompanied by peroxide formation, whereas the oxidation of the natural isomers was not. It was therefore of interest to study further the production of methemoglobin by various tissues with and without the addition of specific substrates. The results show that different tissues vary greatly in their ability to produce methemoglobin from hemoglobin and that cyanide affects this production differently. The reduction of methemoglobin anaerobically can also occur and it was thus possible to show that biological systems can act reversibly upon hemoglobin and methemoglobin.

There is comparatively little literature on the biological production of methemoglobin. Some time ago it was noticed (2) that bacteria grown on media containing blood produced methemoglobin and this was attributed to hydrogen peroxide formation. More recently Amberson and coworkers (3) found methemoglobin in dogs whose blood contained hemoglobin in solution. Other papers on the subject are mostly concerned with the production of methemoglobin in the animal body by drugs such as aniline derivatives, nitrites, and methylene blue.

EXPERIMENTAL

Most of the experiments were carried out in the Barcroft-Warburg apparatus with rat tissues. Preliminary results showed that no methemoglobin was formed from added hemoglobin when

tissue slices were used. This was to be expected, as the diffusion of hemoglobin into the intact cell would, if it occurred at all, be very slow. The tissues therefore were minced, ground in a mortar with sand with 0.05 M phosphate buffer, and except in the case of muscle squeezed through muslin. The results obtained therefore represent the activity of the systems remaining in each tissue after this treatment.

The hemoglobin used was prepared from human blood. The cells were centrifuged and washed, laked with toluene, centrifuged again, and dialyzed against running water in the ice box until no trace of toluene remained. The methemoglobin was prepared from this solution by oxidation with ferricyanide or chlorate and dialyzed to get rid of any excess of these reagents. Varying amounts of hemoglobin or methemoglobin were added to the tissue in the Warburg vessels and the whole volume made up to 2.0 cc. with buffer at the desired pH. At the end of the experiment the contents of the vessels were transferred to centrifuge tubes, 1.5 cc. of saturated ammonium sulfate solution added, and the mixture centrifuged for a short time. This resulted in a clear solution, so that the hemoglobin or methemoglobin could be accurately determined spectrophotometrically. Control experiments showed that this amount of ammonium sulfate did not affect the hemoglobin-methemoglobin ratio.

The methemoglobin was determined by a method which is a modification of the spectrophotometric method suggested by Hüfner (4). This is carried out by measuring the light transmission of the solution containing alkaline methemoglobin and oxyhemoglobin at two convenient wave-lengths, usually 540 $m\mu$ and 560 $m\mu$. Austin and Drabkin (5) have pointed out that if readings were taken at 560 and 575 $m\mu$ a more accurate determination of the methemoglobin could be made, since the relative change in transmission with change in composition is greater at these two wave-lengths than it is at 540 and 560 $m\mu$. A disadvantage of these methods is the fact that the light transmission of methemoglobin varies with the pH and ionic strength.

We have modified this method by first converting all of the methemoglobin to cyanmethemoglobin and then measuring the light transmission at 560 and 575 $m\mu$. Using the ratio of the extinction coefficients at 575 to 560 $m\mu$, the percentage of methe-

moglobin can be read directly from a curve, which was calculated from the extinction coefficients for pure cyanmethemoglobin and pure oxyhemoglobin. The extinction coefficient values used were those given by Austin and Drabkin.

The light transmission of both cyanmethemoglobin and oxyhemoglobin is little affected over the pH range from 6 to 9 and is insensitive to fairly large changes in ionic strength, thus possessing an advantage over previously suggested spectrophotometric methods. A further advantage lies in the large change in the ratio of the extinction coefficients with change in composition of the solution. For pure oxyhemoglobin the ratio is 1.73 and for pure cyanmethemoglobin it is 0.76. The disadvantage of measuring the extinction coefficient of cyanmethemoglobin at 560 and 575 $m\mu$, where the transmission is changing most rapidly with wave-length, was found by actual experiment to be slight.

Hemoglobin alone shaken in air at 37° forms a certain amount of methemoglobin and this is increased somewhat by the presence of cyanide. It was therefore necessary to run a control for each experiment and subtract the amount of methemoglobin formed from hemoglobin alone from that formed from hemoglobin with the tissue. The tissues themselves always contained a small amount of hemoglobin, even though the rats were killed by decapitation and as much blood as possible squeezed out. The amount in them was small, however, compared with the amount of hemoglobin added and the error was thus negligible. This was also true of the heart which contains bound hemoglobin which is readily oxidized to methemoglobin when it is in solution.

The methemoglobin formed when hemoglobin and a tissue are shaken together in air might be produced by several mechanisms. First, the presence of colloidal material might catalyze the oxidation. In order to test this possibility the tissue was boiled. It was found that under these conditions somewhat less methemoglobin was formed in the presence than in the absence of the tissue. The probable reason for this is that the boiled protein contained reducing substances such as sulfhydryl groups which either prevented the methemoglobin from being produced as rapidly as in the control or reduced it once it had been formed. Secondly, it was possible that traces of copper present in the tissues might catalyze the oxidation of hemoglobin, for

copper salts are known to have this property. The experiment
with boiled tissue indicates that traces of copper if present have
little effect. In order to prove this, however, pyrophosphate in
excess was added to unboiled tissue and found to have no effect
on the amount of methemoglobin formed. Thirdly, it was pos-
sible that tissues contain some thermolabile catalyst capable of
oxidizing hemoglobin to methemoglobin. In order to test this
the following experiment was carried out. The tissue suspension
was divided into two equal parts. One part was dialyzed for
24 hours in the ice box; the other placed in a beaker at the same

TABLE I

Methemoglobin Formation by Various Tissues under Different Conditions

The average length of the experiments was 2 hours at 37°. The dialyzed
and non-dialyzed tissues were made up to the same volume. Pyrophos-
phate inhibits the O_2 uptake and this accounts for the somewhat lower
values obtained.

Tissue	Methemoglobin	Methemoglobin	
		0.01 M KCN	0.4 % pyrophosphate
	mg.	*mg.*	*mg.*
Boiled kidney	−2.5	−3.3	
	−2 5	−1.6	
Kidney 24 hrs. old	5.7	8.4	
Dialyzed kidney 24 hrs. old	2.7	2.4	
Liver 24 hrs. old	. 7.6	3.2	
Dialyzed liver 24 hrs. old	0.7	1.0	
Kidney	5.9		4.2
Heart	5.0		5.0
Liver	8.7		4.6

temperature. At the end of this time the volume of the dialyzed
part was measured and the undialyzed part was then made up
to the same volume before being placed in the vessels with the
hemoglobin. The two parts thus contained the same amounts of
non-dialyzable material. The results showed that much less
methemoglobin was formed by the dialyzed portion. The results
of these experiments are shown in Table I. Taken together they
show that the amount of methemoglobin formed by a tissue is a
function of the amount of substrate being oxidized by the tissue;
in other words a function of its metabolism.

Granting this conclusion, it seemed probable that the methemoglobin production was the result of peroxides formed as by-products of the oxidation of certain substrates. From the potentials of the hemoglobin-methemoglobin system and the tissue suspensions it was very unlikely that any direct oxidation could take place. In order to test how quantitative the reaction between hemoglobin and peroxide was the following experiment
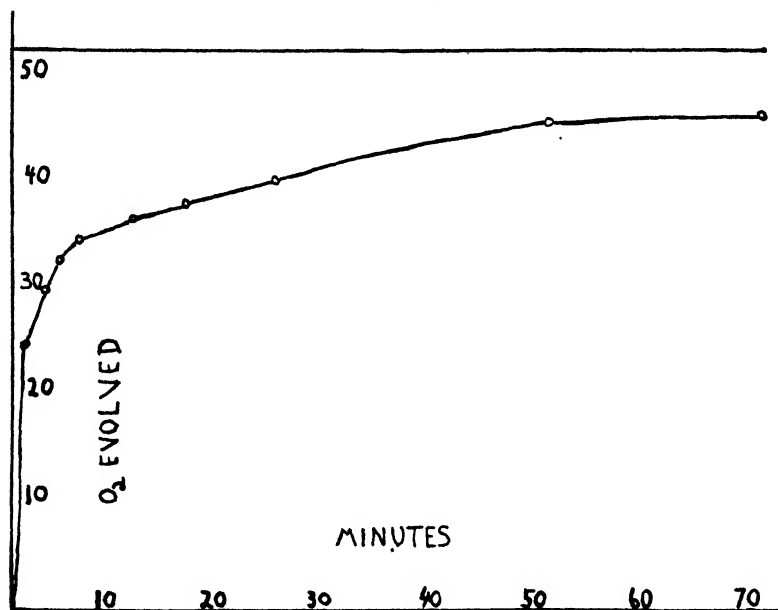
FIG. 1. Oxygen evolution, in c.mm., when H_2O_2 and hemoglobin are mixed in the presence of 0.01 M KCN at pH 6.7 at 37°. The horizontal line represents the amount that should have been evolved calculated from the amount of methemoglobin formed. A similar curve is obtained at pH 7.9.

was done. A known amount of hemoglobin was placed in a Warburg vessel and a known amount of hydrogen peroxide in the side arm. Cyanide was then added to inhibit any catalase activity. The peroxide and hemoglobin were then mixed and the oxygen evolution measured. At the end of the experiment the amount of methemoglobin formed was determined. As shown in Fig. 1, most of the reaction occurs rapidly and 90 per cent

of the theoretical oxygen is given off. It is unaffected by a pH change from 6.7 to 7.9 and by the amount of cyanide present. Because of this oxygen evolution, when methemoglobin is formed from hemoglobin, it was necessary to add to the apparent oxygen uptake of tissue and hemoglobin 1.34 c.mm. of oxygen for every mg. of methemoglobin formed. The presence of hemoglobin or methemoglobin accelerates the oxygen uptake of the tissues to a very small extent. Whether this is due to the pigment or to traces of substances accompanying it has not been determined.

Table II gives average values taken in each case from at least ten individual determinations, showing the amount of methemoglobin formed by various tissues. The kidney is most active

TABLE II

Methemoglobin Formation by Various Tissues at pH 6.7 and pH 7.9 in Presence and Absence of 0.01 M KCN

The average length of the experiments was 2 hours at a temperature of 37°. The figures are average values which are expressed in mg. of methemoglobin per 100 c.mm. of O_2 uptake.

Tissue	pH 6.7		pH 7.9	
	No KCN	0.01 M KCN	No KCN	0.01 M KCN
Kidney	10.9	18.3	10.8	16.1
Brain	2.1	5.8	0.9	1.5
Muscle	1.6	3.5	1.6	2.3
Heart	4.0	0.0	2.7	0.0
Liver	3.6	0.2	2.7	1.8

in this respect, followed by the heart, liver, brain, and muscle in the order named, the difference between the last two being small. Particularly striking, however, are the differences between kidney and liver and between heart and skeletal muscle. The peroxide-forming ability of these tissues must therefore vary considerably, although stoichiometrically 1.0 mg. of methemoglobin represents only about 0.001 mg. of a peroxide of low molecular weight. In order to decide what sort of peroxide was responsible for the methemoglobin formation experiments were done with cyanide. If hydrogen peroxide were formed, then the cyanide in inhibiting catalase and peroxidase ought to increase the yield of methemo-

globin. This actually occurs, as will be shown below, when specific substrates known to produce hydrogen peroxide are added to the tissue. The final concentration of the cyanide was 0.01 M, which was sufficient to cause an inhibition of the tissue respiration throughout the experiment despite the fact that as methemoglobin was formed cyanide was used to form cyanmethemoglobin. Thus there was 10 times more cyanide present than was necessary to combine with the maximum amount of methemoglobin formed. Cyanide never produced complete inhibition. It was thus possible to calculate the methemoglobin formed per 100 c.mm. of oxygen uptake. The results are shown in Table II. With the kidney the addition of cyanide definitely increases the methemoglobin formation per 100 c.mm. of oxygen uptake and may increase it absolutely. With muscle and brain the absolute amount of methemoglobin formed in the presence of cyanide is somewhat less than in its absence, but per 100 c.mm. of oxygen uptake the amount formed is always greater with cyanide. But with liver and heart cyanide definitely inhibits methemoglobin formation. These differences do not seem to be correlated with the percentage inhibition of oxygen uptake caused by the cyanide on the different tissues. Thus the average inhibitions for the two hydrogen ion concentrations given in Table II were kidney 32 per cent, brain 47 per cent, muscle 51 per cent, heart 52 per cent, and liver 47 per cent. Although in the two tissues in which cyanide inhibits methemoglobin formation the inhibition of oxygen uptake is largest, the differences in the cyanide sensitivity are not large enough to account for the differences in methemoglobin formation. Changing the relative concentrations of cyanide, hemoglobin, and tissue did not alter these effects. Therefore the peroxide-forming mechanisms in liver and heart must be considered cyanide-sensitive and thus different from those in the kidney, brain, and muscle.

Changing the concentration of the tissue and keeping the hemoglobin and cyanide constant alter the amounts of methemoglobin produced and this is proportional within limits to the amount of tissue present and the oxygen uptake. It was possible, however, that methemoglobin was being reduced aerobically by substances in the tissue and that the amount formed at the end was the algebraic sum of the oxidizing and reducing power of the tissue. To

test this point methemoglobin was added to tissues and shaken in the Warburg vessels, but it was found that no reduction had taken place with or without cyanide, except in the case of liver which showed in certain experiments a slight reduction in the presence of cyanide.   It is possible to assume therefore that the methemoglobin once formed remains as such until the end of the experiment.

The effect of pH on the methemoglobin production can be seen in Table II.   In general more is produced per 100 c.mm. of oxygen uptake at pH 6.7 than at pH 7.9.   The rate of oxygen uptake

TABLE III

Methemoglobin Formation in Kidney and Liver in Relation to Oxygen Uptake.   pH 6.7 at 37°

Tissue	Time	O₂ uptake	MetHb formation	MetHb per 100 c.mm. O₂ uptake	0.01 M KCN		
					O₂ uptake	MetHb formation	MetHb per 100 c.mm. O₂ uptake
	min.	*c.mm.*	*mg.*	*mg.*	*c.mm.*	*mg.*	*mg.*
Kidney	30	38	0.8	2.1	22	2.1	9.5
	60	79	4.1	5.2	29	4.5	15.5
	120	141	8.2	5.8	42	6.2	14.8
	180	215	11.5	5.3	43	7.4	17.2
Liver	30	66	0.9	1.4	31	−2.1	0.0
	75	101	4.0	4.0	41	−2.7	0.0
	145	148	7.6	5.1	71	−3.7	0.0
	195	189	11.0	5.8	74	−4.0	0.0

is generally somewhat greater at the lower than at the higher pH and these two facts may be correlated.   It is also true that sulfhydryl groups whether attached to proteins or not are more oxidizable in alkaline solutions and it seems probable that some of the peroxide formed under these conditions may be used in oxidizing these groups; in other words, another factor is introduced to compete with the hemoglobin for the peroxide.

Table III shows the production of methemoglobin with time by kidney and liver.   Warburg vessels were set up with the tissue alone and with 0.01 M cyanide and at intervals a pair was removed and the methemoglobin production determined.   In this way the methemoglobin and oxygen uptake could be correlated.

Except in the first 30 minutes, when the production of methemoglobin lags, the amount produced per 100 c.mm. of oxygen uptake remains fairly constant for the rest of the experiment. This is further evidence that methemoglobin production is a function of the oxidative metabolism. In this experiment the liver in the presence of cyanide produced less methemoglobin than the blood and cyanide alone. This inhibition of methemoglobin formation is comparatively small and it is not possible to account for the cyanide effect with this tissue on the assumption that methemoglobin production is completely inhibited or that it is being reduced as rapidly as it is formed.

In order to test the effect of added substrates on the methemoglobin formation tyramine and l-alanine were chosen, for both have been shown (6, 7) by different methods to produce hydrogen peroxide during oxidation. The catalysts responsible for the oxidation of these substances are cyanide-insensitive, so it was possible to use cyanide in the attempt to get theoretical yields of methemoglobin on the assumption that hydrogen peroxide is quantitatively produced during the oxidation. Preliminary experiments showed that when these substances were oxidized, more methemoglobin was produced than in the control and that cyanide greatly increases the difference. Thus in one experiment with kidney without cyanide when 0.1 mg. of l-alanine was oxidized, 0.7 mg. more methemoglobin was produced than in the control. In another experiment without cyanide with dialyzed liver 2.8 mg. more methemoglobin were formed when 0.2 mg. of· tyramine was being oxidized than in the control. These experiments are important because they show that the hemoglobin can compete with catalase and other substances and explain why methemoglobin can be formed in tissues by hydrogen peroxide in the absence of cyanide. Hydrogen peroxide added in excess to tissue and hemoglobin will also produce methemoglobin in the absence of cyanide.

For quantitative results, however, cyanide must be used. 0.1 mg. of l-alanine oxidized by kidney in the presence of 0.01 M cyanide produced 28 mg. more methemoglobin than the control. This corresponds to 0.028 mg. of hydrogen peroxide. Theoretically this amount of alanine should produce 0.038 mg. The yield was thus 74 per cent. In this experiment the minimum

amount of kidney was used. In the presence of more kidney the yield is cut down and may under certain conditions be as low as 40 per cent. In the case of 0.2 mg. of tyramine oxidized by liver in the presence of 0.01 M cyanide 20.5 mg. more methemoglobin was formed than in the control. This corresponds to 0.021 mg. of hydrogen peroxide. The theoretical amount of hydrogen peroxide was 0.049 mg., giving a yield of 43 per cent. These yields indicate that in the absence of cyanide the methemoglobin production represents only about 10 per cent of the peroxide formed by tissues and in the presence of cyanide only about 50 per cent. An attempt was made to see whether it was possible to detect peroxide formation when succinic acid was oxidized,

TABLE IV

Anaerobic Reduction of Methemoglobin

Dialyzed liver was used at pH 7.5. The methemoglobin was estimated after 2 hours at 37°. The molar concentration of the methemoglobin corresponded to that of a 1:5000 methylene blue solution.

Buffer	Liver	Succinate (2 mg.)	Choline (2 mg.)	MetHb (8.3 mg.)	Hb formed	Reduction	Reduction of 1 cc. methylene blue
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*mg.*	*per cent*	*min.*
1.4	0.5			1.0	2.1	22	80
1.0	0.5		0.4	1.0	3.4	36	17
1.0	0.5	0.4		1.0	2.5	27	5
1.9				1.0	0.0	0	

because the succinoxidase unlike the tyramine and *l*-alanine oxidases is cyanide-sensitive. Although the conditions were varied in every possible way, no extra methemoglobin was formed when succinic acid was oxidized. If peroxide is formed by this system, it cannot be detected by the hemoglobin method.

Anaerobically all the tissues tried reduce methemoglobin, although the reduction is considerably slower than that of methylene blue of the same molar concentration. The methemoglobin was made up so that its molarity was the same as a 1:5000 solution of methylene blue. The usual Thunberg technique was used. Two specific substrates were added, succinic acid and choline, and the rates of reduction of methylene blue and methemoglobin were compared. A typical experiment is shown in

Table IV. Inasmuch as the potentials of the methylene blue and the methemoglobin are almost the same, the difference in reduction rate must be due to differences in availability of these substances to the enzyme surface.

It is shown from the above experiments that the methemoglobin formation is a function of the oxidative activity of the tissue. In any one tissue the methemoglobin increases with the oxygen uptake but the ability of different tissues to produce methemoglobin under the experimental conditions varies greatly. If, as it seems probable, peroxide formed as a by-product to various oxidations is the cause of the methemoglobin production, then there should be some correlation between cyanide sensitivity and methemoglobin formation, because it is only in the cyanide-insensitive systems that peroxide formation has been definitely proved. Such a correlation does not hold and, moreover, the fact that methemoglobin production is suppressed in heart and ·liver by cyanide makes the interpretation of these results difficult. It is possible that cyanide-sensitive systems also produce peroxide but the evidence thus far obtained by the hemoglobin method is against this, for succinoxidase, d-alanine oxidase, and choline oxidase, which are all cyanide-sensitive, show no methemoglobin production. The possible formation of peroxides other than hydrogen peroxide as suggested by Keilin and Hartree (8) may account for the methemoglobin production in the absence of cyanide if such peroxides are not attacked by catalase but does not account for the cyanide effect in heart and liver.

The presence of hemoglobin or methemoglobin has only a very slight effect on the oxygen uptake of the tissue, so the pigment is not directly involved as a catalyst in the oxidation nor does the presence of the pigment affect the cyanide sensitivity of the tissue. On the other hand, methylene blue added to tissues under the same conditions greatly increases the oxygen uptake. It seemed possible that peroxide formed by the residual oxygen uptake would accumulate in the presence of cyanide because of the inactivation of the catalase and add its toxic effect to that of the cyanide. The presence of the hemoglobin would remove the peroxide and thus diminish the apparent cyanide sensitivity. This, however, was not the case.

It was possible that, as methemoglobin was formed during the oxidation, it would combine with peroxide to form a complex, as shown by Keilin and Hartree (8). This complex is very unstable and breaks down with the destruction of some of the methemoglobin. If this happened, there should be a progressive loss of this pigment as the oxidation proceeded. But as the methemoglobin production parallels the oxygen uptake, such complexes if formed play no significant part. Finally, phosphate, which affects cyanide sensitivity according to Alt (9), was used throughout these experiments but always in the same concentration, so that this factor cannot account for the observed differences in the methemoglobin production by various tissues.

SUMMARY

1. The production of methemoglobin from hemoglobin by a tissue suspension is a function of the oxygen uptake. Traces of copper, if present, or inactive colloidal material play no part in this reaction.

2. Per 100 c.mm. of oxygen uptake the kidney produces most methemoglobin, followed by heart, liver, brain, and muscle in this order.

3. Cyanide increases the amount of methemoglobin formed per 100 c.mm. of oxygen uptake in kidney, brain, and muscle but decreases it in liver and heart.

4. Tyramine and l-alanine, which are known to produce hydrogen peroxide when oxidized, increase the methemoglobin production and give fair yields on the assumption that hydrogen peroxide is quantitatively formed during the oxidation. Succinic acid, d-alanine, and choline produce no extra methemoglobin.

5. Methemoglobin is reduced anaerobically but not aerobically. Addition of succinic acid or choline increases the amount of reduction, but the rates are much slower than when an equivalent quantity of methylene blue is reduced.

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THE EFFECT OF METAPHOSPHORIC ACID AND SOME OTHER INORGANIC ACIDS ON THE CATALYTIC OXIDATION OF ASCORBIC ACID*

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The instability of ascorbic acid in weakly acid solutions has recently been shown to be due to the copper content of the solutions (1). By strict elimination of copper, solutions of ascorbic acid are stable below pH 7.6. The preparation of copper-free reagents, however, is not very practical and in analytical procedures would not eliminate copper contamination from the materials analyzed. The use of metaphosphoric acid for the extraction and titration of ascorbic acid has resulted in the stabilization of ascorbic acid solutions so treated (2–4). Recently diethyldithiocarbamate (5), 8-hydroxyquinoline, glutathione (6), and other compounds forming copper complexes (7) have been used for the stabilization of ascorbic acid solutions.

The work reported here was undertaken primarily to investigate a possible quantitative relationship of ascorbic acid, copper, and the amount of metaphosphoric acid necessary to inhibit atmospheric oxidation. A few related observations are also reported.

EXPERIMENTAL

All experiments were carried out in a Warburg respirometer at 37.5°. The water used for making solutions and rinsing apparatus was distilled twice in Pyrex all-glass stills. The solutions of ascorbic acid were made from crystalline ascorbic acid and their

FIG. 1. Effect of the ratio HPO$_3$:Cu on the oxygen uptake of ascorbic acid. The acidity of the solutions was the same in all cases, 4×10^{-5} mole of HPO$_3$; pH 2.16; ascorbic acid 8×10^{-6} mole; amount of copper (varied) 1 to 10×10^{-6} mole, depending upon the ratio HPO$_3$:Cu, as indicated on each line.
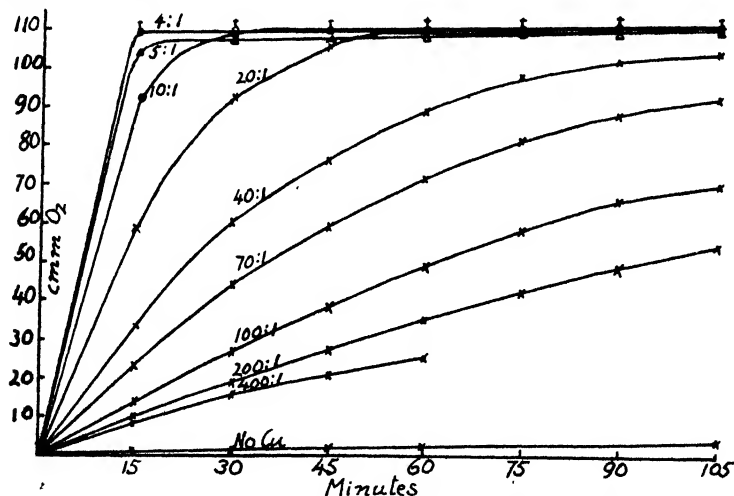
FIG. 2. Effect of the ratio H$_2$SO$_4$:Cu on the oxygen uptake of ascorbic acid. The acidity was the same in all cases, 2×10^{-5} mole of H$_2$SO$_4$; pH 2.18; ascorbic acid 8×10^{-6} mole; amount of copper (varied) 1×10^{-7} to 1×10^{-5} mole, depending upon the ratio of replaceable hydrogen of H$_2$SO$_4$:Cu, as indicated on each line.

strength was checked at the start of the experiments by iodine titration. The solutions of metaphosphoric acid were prepared immediately before each experiment by dilutions from a known weight of metaphosphoric acid, their strength being adjusted, however, by titration with standard NaOH with methyl red as indicator. Depending upon the purpose of the experiment,
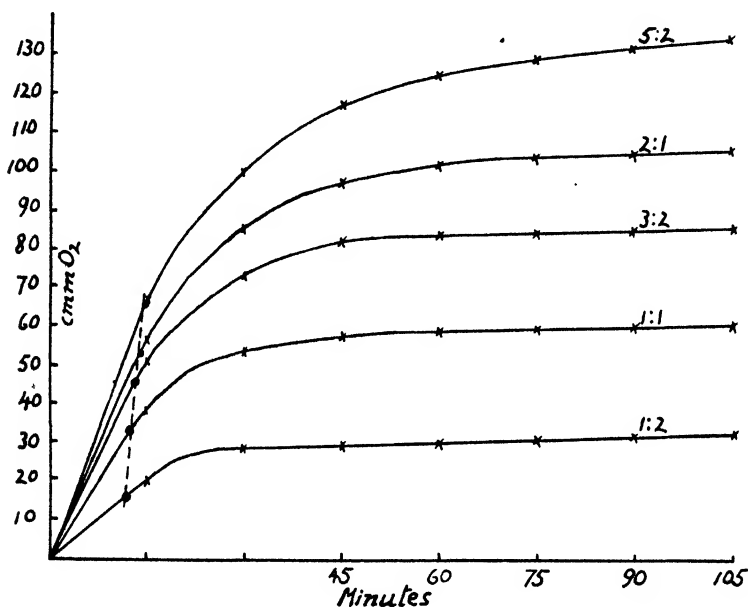
FIG. 3. The effect of different ratios of ascorbic acid to HPO_3 on the rate of oxidation of ascorbic acid in the presence of copper. In each case the concentration of HPO_3 was 4×10^{-6} mole, that of Cu 1×10^{-6} mole. The concentration of ascorbic acid varied from 2×10^{-6} mole to 1×10^{-5} mole and the acidity of the solutions from pH 3.00 to 3.10. The broken line passes through the time of half oxidation. The ratio of ascorbic acid to HPO_3 is indicated on each line.

either the copper ($CuSO_4$) or the ascorbic acid was added from the side arm to the contents of the respirometer flasks after equilibration and closing the taps.

Typical results obtained with metaphosphoric acid are illustrated in Fig. 1. When they are compared with the results in Fig. 2, it is evident that the inhibitory action of metaphosphoric acid depends upon a lowering of the pH of the solution and, in

addition, a depression of the copper ion concentration. The rate of oxidation in the presence of metaphosphoric acid depends, as in the presence of sulfuric acid, on the amount of copper present. More copper is required, however, to obtain a given rate of oxidation in a metaphosphoric acid solution than in a sulfuric acid solution. There is no definite stoichiometic relation between the molar quantities of metaphosphoric acid and of copper at which copper catalysis is completely inhibited and which might point to the formation of a completely undissociated copper complex. In this respect the action of metaphosphoric acid is different from that of some of the organic compounds that have been used to inhibit the copper-catalyzed oxidation of ascorbic acid (6–7). The results illustrated in Fig. 1 were obtained with a sample of purified metaphosphoric acid (titrating 95 per cent HPO_3) at pH 2.16.[1] At other pH values (below pH 3.5) and with Mallinckrodt's c.p. HPO_3 (titrating 40 per cent HPO_3), similar results were observed; *i.e.* in all cases the extent to which copper catalysis was inhibited depended upon the molar ratio HPO_3:Cu.

Fig. 3 illustrates the fact that the molar ratio, ascorbic acid to HPO_3, has no effect on the rate of oxidation of ascorbic acid in the presence of copper. This indicates that the inhibiting effect of metaphosphate is due to its action on copper and not on the ascorbic acid.

In enzymatic studies involving ascorbic acid, in which buffered solutions must be used, the oxidation of ascorbic acid due to copper contamination of the buffers is of considerable importance. We investigated, therefore, the possibility of using neutralized metaphosphoric acid in the buffers in an attempt to stabilize ascorbic acid. The acid was readily oxidized in such solutions even when the molar ratios PO_3^-:Cu^{++} were as high as 1000:1. In buffer solutions metaphosphate is therefore of no value in stabilizing ascorbic acid.

The oxidation of ascorbic acid to dehydroascorbic acid involves two adjacent hydroxyl groups. Since Boeseken's work on the increased conductivity of boric acid in the presence of *cis* glycols suggests the formation of a complex between the acid and the glycol, it was considered possible that boric acid would inhibit

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[1] We are indebted to Dr. E. P. Partridge of the Hall Laboratories, Pittsburgh, for this sample of metaphosphoric acid.

the copper-catalyzed oxidation of ascorbic acid.   Experiments failed to substantiate this to a significant degree.   Although the oxidation of ascorbic acid in boric acid solutions and in borate buffers was very slow, addition of small amounts of copper to such solutions resulted in rapid oxidation.

Since metaphosphoric acid has such a marked inhibitory effect on the copper-catalyzed oxidation of ascorbic acid, a few comparative observations were made with other inorganic acids.   In these
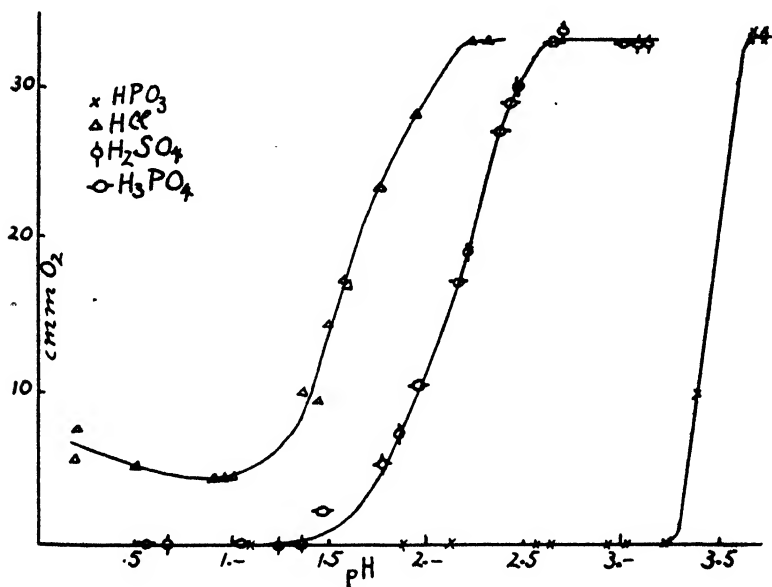
FIG. 4. A comparison of the oxygen uptake by ascorbic acid in the presence of copper in different acids.   In all cases the concentration of ascorbic acid was 2×10^{-6} mole; that of copper ($CuSO_4$) 2.5×10^{-7} mole.

experiments the concentrations of ascorbic acid (2×10^{-6} mole) and of added copper (2.5×10^{-7} mole) were maintained constant while the pH of the solutions was varied.   Fig. 4 illustrates the results with HPO_3 (c.p. sticks), H_2SO_4, H_3PO_4, and HCl.   c.p. H_3PO_4 gave erratic results until it was purified by precipitation with $Ba(OH)_2$, washing with glass-distilled water, and decomposition of the barium phosphate with H_2SO_4 (avoid an excess). HCl was prepared from c.p. NaCl and H_2SO_4; HBr was made from

bromine and phosphorus in an atmosphere of CO_2. In all cases the acids were dissolved in glass-distilled water and precautions were taken to minimize copper contamination. The results of these experiments again illustrate the protective action of metaphosphoric acid. In orthophosphoric and sulfuric acid solutions the rate of oxidation of ascorbic acid is strictly a function of the hydrogen ion concentration and is apparently not affected by the anion, since the curves obtained with the two acids are identical. In a medium of halogen acid, however, ascorbic acid is oxidized at an appreciable rate, even in very acid solutions. As the acidity increases a point of minimal oxidation is reached (about pH 1) beyond which oxidation again becomes more rapid. Similar results were obtained with HBr. Evidently it is not advisable to use HCl for extraction and titration purposes, as suggested by Bukin (8). It must be emphasized that the curves illustrated in Fig. 4 hold only for solutions having the copper concentrations used in these experiments. They illustrate, however, the relative effectiveness of the various acids in suppressing the copper-catalyzed oxidation of ascorbic acid.

Recent work by Borsook and associates (9) indicates that below pH 4.5 oxidation of ascorbic acid proceeds only to the reversible oxidation product, while at higher pH values non-oxidative and oxidative irreversible changes take place. The oxidation of ascorbic acid at pH 4.5 should therefore require 1 atom of oxygen per mole of ascorbic acid. In our experiments we observed a considerably higher oxygen consumption. Barron *et al.* (1) have postulated that H_2O_2 is formed during copper-catalyzed oxidation of ascorbic acid and that the peroxide formed is decomposed as rapidly as it is formed. Incomplete decomposition of H_2O_2, however, could explain the high oxygen consumption that we observed. Qualitative tests (10) readily indicated the presence of H_2O_2. Attempts to estimate H_2O_2 quantitatively by iodometric titrations (11, 12) failed to give consistent results, because the copper contained in the ascorbic acid solutions greatly accelerated the liberation of iodine from KI and thus made the titrations uncertain. The oxygen uptake beyond that calculated for the reaction, ascorbic acid $\overset{(O)}{\rightarrow}$ dehydroascorbic acid, was not due to further oxidation of dehydroascorbic acid to an irreversible oxidation product, because treatment of the oxidized solutions with

H_2S gave complete recovery of the ascorbic acid. Hence the excess oxygen uptake observed in experiments carried out below pH 4.5 is undoubtedly due to formation of H_2O_2.

Molybdic acid has been used by Stern (11) to speed up the decomposition of H_2O_2. Attempts to use this as a means of arriving at the theoretical oxygen uptake failed, however, because solutions of ascorbic acid containing molybdic acid turn blue, owing to reduction of the latter by ascorbic acid.

In buffered solutions of pH 4.5 the oxidation of ascorbic acid in the presence of copper proceeds rapidly until an oxygen uptake somewhat higher than that calculated for the reaction ascorbic acid $\overset{(O)}{\rightarrow}$ dehydroascorbic acid is reached. Then oxidation continues at a much slower rate. During this second stage of the oxidation, evolution of CO_2 can be demonstrated in parallel experiments with and without provision for the absorption of CO_2. It is particularly marked in McIlvaine's citrate-phosphate and in Sørensen's phosphate buffers, while in phthalate-NaOH buffers (13) it is only slightly noticeable. These observations indicate that copper catalysis accelerates not only the reversible oxidation of ascorbic acid but also further oxidation of dehydroascorbic acid. This emphasizes the necessity of using inhibitors of copper catalysis when ascorbic acid is used in experiments involving buffer solutions.

SUMMARY

1. Metaphosphoric acid inhibits the copper-catalyzed oxidation of ascorbic acid by decreasing the amount of copper effective in the catalysis, in addition to the effect that is due to the pH of the solution.

2. The rate of oxidation of ascorbic acid in solutions of metaphosphoric acid depends primarily upon the ratio $HPO_3:Cu$, rather than the ratio of HPO_3 to ascorbic acid.

3. In buffered solutions near pH 7 metaphosphate does not appreciably inhibit the oxidation of ascorbic acid.

4. In acid solutions containing the same amount of copper, the rate of oxidation of ascorbic acid depends entirely upon the hydrogen ion concentration in the case of sulfuric and orthophosphoric acids, while in hydrochloric and hydrobromic acids it is also affected by the anion (accelerated).

5. Atmospheric oxidation of ascorbic acid in the presence of copper leads to the formation of hydrogen peroxide and, in solutions of pH > 4.5, to the evolution of CO_2.

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BIOCATALYTIC ACTIVATORS SPECIFIC FOR THE YEAST FERMENTATION OF MALTOSE*

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It has long been the generally prevailing belief that maltose is fermentable by yeast only after hydrolytic conversion to glucose by the enzyme maltase present either in the yeast or in the substrate, or both. This view-point has, however, been challenged by certain workers, including Willstätter and Stiebelt (1), Willstätter and Bamann (2), and Sobotka and Holzman (3), whose observations indicate that preliminary hydrolysis to glucose is not necessarily an essential requirement in maltose fermentation.

Yeasts show a wide range of maltase activity, and maltase is considered to be extremely unstable. Harding and Nicholson (4) reported that bakers' yeast loses its maltase activity almost completely after standing a few days at room temperature. Sandstedt and Blish (5) found bakers' compressed yeast frequently deficient in its ability to ferment *pure maltose*, and that this ability decreased with age and with elevation of storage temperature. They also found, however, that the lack or the loss of the yeast's power to ferment *pure maltose*, alone, did not significantly impair its ability to ferment maltose as produced by diastasis in wheat flour paste or dough.

Using a special manometric technique, Sandstedt and Blish (5) showed repeatedly that in a diastatically active flour-water paste containing 3 to 5 per cent of yeast the rate of gas production was the same for 15 to 20 day-old samples of yeast as for a fresh sample.

The deficiency frequently shown by bakers' yeast in fermenting pure maltose, when acting upon it alone, in contrast to its ability to ferment maltose without difficulty when in contact with a diastatically active flour paste or dough, permits only one reasonable conclusion.   The flour must have furnished a factor or combination of factors without which the yeast could not ferment maltose effectively.   Tests for maltase activity in the flour, alone, gave negative results.   A sample of flour was then rendered enzymatically inert by suspension for 1 hour in dilute HCl, followed by washing with strong alcohol, neutralization, and drying.   This flour, when doughed with an old yeast, showed completely negative fermentation.   Fermentation was, moreover, so slight as to be almost negligible in the case of pure maltose alone, in contact with the yeast.   Nevertheless, an active fermentation occurred when the same yeast was added to a mixture of the inactivated flour and pure maltose.

It was concluded by Sandstedt and Blish (5) that flour contains a yeast maltase stimulant or activator that is non-enzymic.   However, it would seem equally plausible to consider that the activator is not necessarily a *maltase* stimulant, but that it is possibly, if not probably, a factor that induces yeast to ferment maltose directly, and without any preliminary hydrolysis to glucose.   Regardless of how it functions, it serves effectively as a maltose fermentation activator or accelerator.

More recently, Genevois and Pavloff (6) also have reported the existence of a maltose fermentation catalyst in flours.   They find that the amount varies with different flours, and conclude that the catalyst is probably identical with Factor Z of von Euler and Swartz (7).

The literature of recent years has recorded an enormous volume of research on yeast fermentation as influenced by a variety of catalytic factors including enzymes, coenzymes, non-enzymic accelerators, and inhibitors, among which are salts and other specific substances, some of which have not been identified.   In most instances the effects of these factors have been measured and recorded in terms of CO_2 produced in the fermentation of *glucose* or *sucrose*.

The chief purpose of this communication is to report our further experiences and observations as to the occurrence and properties

of biocatalysts that are seemingly distinctive for their stimulating influence on maltose fermentation.

The method used in these experiments for studying rate and degree of fermentation is essentially a manometric procedure in which the fermentations were conducted in aluminum cups of definite volume, submerged in a water bath at 30°.   Each cup is fitted with a gas-tight lid carrying a mercury manometer, and fermentation rate is recorded in terms of mm. of pressure read at definite time intervals.   Conditions and specifications for securing trustworthy results by this convenient procedure have been described by Sandstedt and Blish (5).

The usual practise in these studies was to use 0.3 to 0.5 gm. of bakers' compressed yeast and 0.3 gm. of maltose,[1] and all experiments involved these amounts unless otherwise specifically stated. The substance or preparation suspected of containing activator was superimposed on this mixture, the total volume of which was seldom more than 5 to 6 cc.   This small volume reduces to a negligible amount the error arising from the solubility of CO_2 in the liquid medium under pressure.

Fermentation of Pure Maltose, Alone, by Bakers' Yeast—The rate and the completeness of fermentation of pure maltose, alone, by a well known brand of bakers' compressed yeast have been found to vary with different lots of yeast.   With some lots there was only a negligible fermentation of the maltose, while others fermented it more completely.   In nearly all instances a state of active fermentation was never reached until after an induction period of many hours.   With all lots the fermentation efficiency was very low as compared with the action of the same yeasts on either glucose or sucrose.   A typical instance of this is shown in Fig. 1.

Fig. 1 shows that the fresh compressed bakers' yeast was very deficient in its ability to ferment pure maltose, alone, as compared to sucrose.

Occurrence of Activator in Flour—That wheat flour contains a non-enzymic maltose fermentation catalyst has been indicated,

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[1] c.p. maltose hydrate was supplied by the Pfanstiehl Chemical Company.

and it is of interest to know whether different flours contain the factor in equal or varying amounts. Accordingly four flours were selected, including two bakers' flours milled from hard winter wheat, one soft wheat flour, and one malt flour milled from germinated wheat. In each case the flour was extracted for 10 minutes with 3 parts of water (preliminary tests having clearly shown the flour activator to be water-soluble). In 25 cc. of extract 1 gm. of yeast was suspended, and the mixture was allowed to ferment overnight at 30° to eliminate fermentable sugars present as such in the extract. Next morning a 7.5 cc. aliquot of the sus-
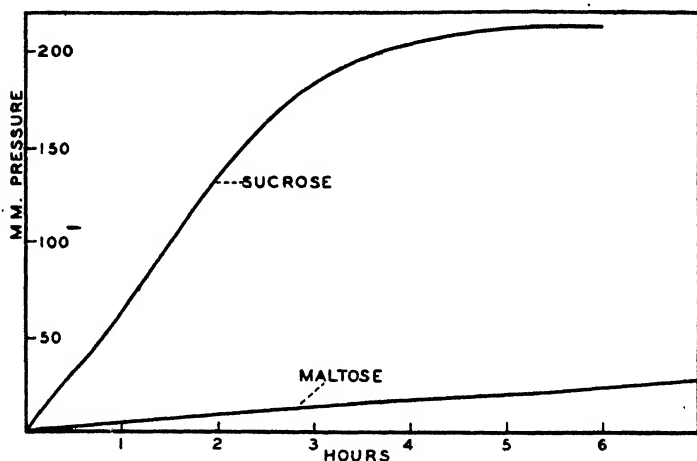
FIG. 1. Comparative fermentabilities of pure maltose and sucrose by fresh bakers' yeast.

pension (equivalent to 2.5 gm. of flour and 0.3 gm. of yeast) was added to 0.2 gm. of pure maltose, and the rate of fermentation was studied by the usual manometric procedure. Blank determinations without maltose gave negligible values. The results are shown graphically in Fig. 2.

From the comparative fermentation rates shown in Fig. 2, it is apparent that flours vary appreciably as to the quantity or potency of activator, as also reported by Genevois and Pavloff (6), and it is evident that malted wheat flour contains much more of it than ordinary flour. In the case of the malted flour activator

the maltose was completely fermented in 5.5 hours, whereas in all other instances fermentation was incomplete even after 8 hours.

Some Properties of Activator As Found in Malted Wheat Flour—Some difficulties were experienced in attempts to prepare the malt flour activator in highly concentrated form, reasonably free from contaminating substances, for the purpose of determining its properties.   The best preparation was one obtained by extracting malted wheat flour with dilute HCl at pH 2 (the purpose in using acid was to destroy enzymic activity), neutralizing the acid, centrifuging, and adding alcohol to about 75 per cent concentra-
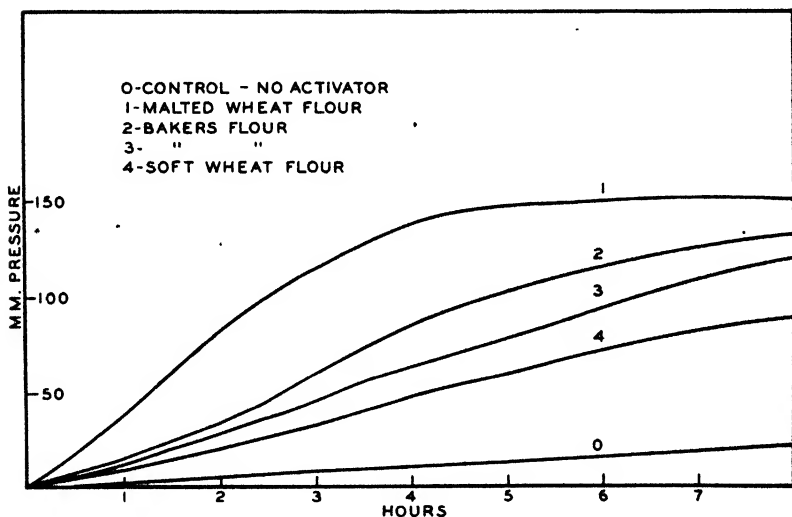
FIG. 2. Comparative amounts of activator in different flours

tion.   The filtrate from the alcohol precipitation was evaporated under reduced pressure, taken up with a little water, and treated with a large excess of acetone.   The precipitate, after drying with acetone and ether, contained a large amount of activator, together with a considerable quantity of fermentable substance. No convenient method of separating the activator from these and other contaminating materials was found, although we have made no serious or systematic effort to isolate and identify the active substance.

Results of a few experiments intended to disclose some of the

more important properties of the activator, as found in wheat flour, can be briefly summarized as follows: It is soluble in water. It is not effectively extracted by alcohol, although in water solution alcohol does not precipitate it until an alcoholic concentration of approximately 90 per cent by volume is reached. It is readily precipitated by acetone. It was found to be completely stable to exposure for 20 minutes in an autoclave at 15 pounds of steam pressure. After dialysis overnight in tap water the activity was greatly reduced, indicating that the factor is dialyzable and therefore presumably of relatively small molecular size. The activator, in water solution, showed evidence of being strongly adsorbed by colloidal ferric hydroxide. These properties are indeed suggestive of Factor Z of von Euler and Swartz (7), as noted by Genevois and Pavloff (6).

Occurrence of Activators in Yeasts and Yeast Preparations—We have made no serious effort to determine the extent to which maltose fermentation activators occur in biological materials of widely different type and origin. We did find appreciable amounts in navy beans, in young wheat leaves, in horse serum, and in eggs. Of the materials thus far studied, certain dried yeast and zymin preparations have afforded the most convenient and effective sources of the activating factor or combination of factors. Several commercial samples of dried yeasts were tried (two were brewers' yeasts and another supposedly dried bakers' yeast) and all were found to be effective, but in varying degrees.

One of the most potent sources of activator was afforded by drying some ordinary bakers' compressed yeast which in its fresh condition was conspicuously lacking in ability to ferment pure maltose. The addition of a small quantity of the dried yeast enabled the fresh yeast to ferment pure maltose rapidly and almost completely. Within certain limitations the method of drying the yeast was not a factor of critical importance.

A commercial sample of zymin (Eimer and Amend) was found to be highly potent. Among the less effective of the yeast preparations were those made by autolysis processes, including treatments, respectively, with toluene, chloroform, acetone, ethyl acetate, etc.

Fig. 3 shows comparative stimulating effects of several dried yeast preparations. In each case the fermenting medium con-

sisted of 0.3 gm. of maltose, 0.3 gm. of fresh yeast, and 0.3 gm. of the activator substance, with 5 to 6 cc. of water. The fermentations were conducted in a 30° water thermostat. It was, of course, necessary to run a blank determination on each preparation, with maltose left out of the mixture.
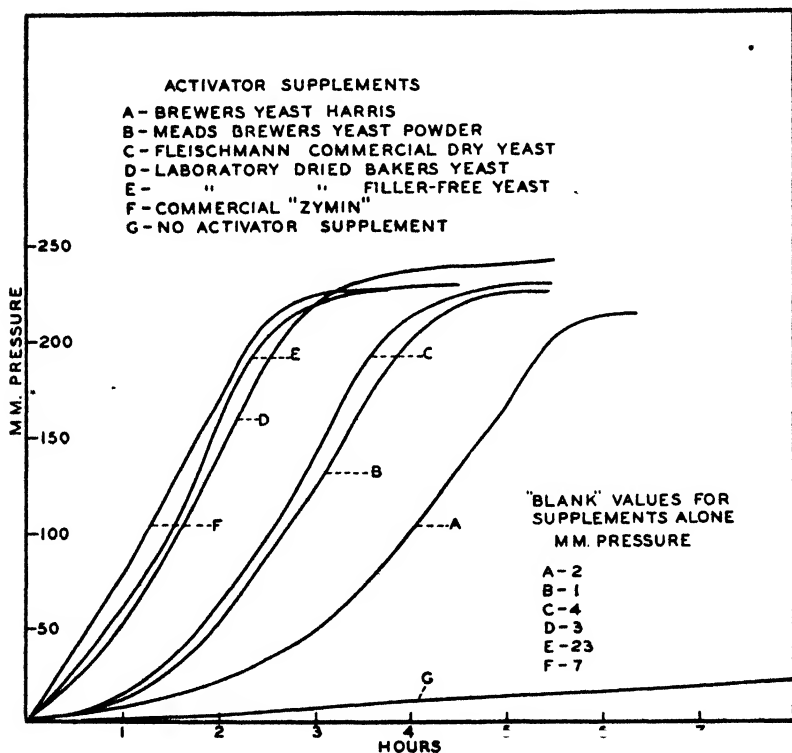
FIG. 3. Acceleration of maltose fermentation by dried yeast preparations

A value of 225 mm. of mercury (after subtraction of the blank) is the highest obtained from 0.3 gm. of maltose in any of these experiments, and this value may be conveniently designated as the optimum yield of carbon dioxide.

From an inspection of Fig. 3 it is evident that the fresh yeast was able to ferment pure maltose actively and readily only when supplemented by some stimulating substance contained in the

dried yeast preparations. When corrections for the blank values are applied, all *combinations* gave the optimum yield in 5 to 6 hours with the single exception of Supplement A, which fell only slightly short.

The significant variable among the different dried yeast products is the induction period, or the time interval elapsing before active fermentation begins. With Supplements A, B, and C (Fig. 3) fermentation did not reach the active stage until approximately 1½ hours had passed, whereas with Supplements D, E, and F active

TABLE I

Fermentation of Pure Maltose by Dried Yeast Preparations, Alone, As Compared with Fresh Yeast Alone, and with Fresh and Dried Yeast Combined

CO_2 pressures are measured in mm. of Hg.

Preparation	CO_2 pressures at successive time intervals						
	2 hrs.	5 hrs.	8 hrs.	12 hrs.	19 hrs.	24 hrs.	48 hrs.
A. Fresh yeast alone....................	9		25		30		
B. Brewers' Yeast-Harris................	2				11	11	128
C. Mead's brewers' yeast powder........	1				13	24	113
D. Dried bakers' yeast (filler-free).......	2		16	34		200	
E. Commercial zymin....................	3		7	10	20	24	205
F. Zymin 1*............................	3		11	23		26	93
" " 2*............................	4		10	22		25	196
A+D. Fresh yeast supplemented by dried yeast................................	203	219					

* The acetone-dried preparations were kindly furnished by Dr. E. I. Fulmer, Iowa State College, Ames.

fermentation began without appreciable delay. Once active fermentation had started, the fermentation rates of Supplements B and C were not significantly different from those of Supplements D, E, and F. This suggests the probable existence of more than one stimulating factor, one of which is effective in eliminating or greatly reducing the induction period, while the other is concerned with the maintenance of a reasonably rapid rate of fermentation after the process is once initiated.

The blank values, with the exception of the laboratory-dried bakers' yeast, were small. That the high blank value for the laboratory-dried bakers' yeast was due to the filler ordinarily

contained therein is evidenced by the fact that a much smaller blank was obtained in the case of some specially purchased filler-free bakers' yeast.

Is the stimulating effect of the dried yeast preparations distinctly a *supplementary* effect, or will these preparations, by themselves, actively ferment pure maltose? In Table I are presented data showing typical observations of the rates at which several of these preparations, respectively, ferment pure maltose *by themselves*, as compared with their activities when used in conjunction with fresh yeast.

The data in Table I show that the efficiencies of the dried yeast products, respectively, in fermenting pure maltose were similar

TABLE II

Maltose Fermentation Rates As Influenced by Varying Amounts of Activator
CO_2 pressures are measured in mm. of Hg.

Activator		CO_2 pressures at successive time intervals					
		½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.
	gm.						
None		5	7	9	13		22
A. Commercial zymin	0.2	38	73	177	220		222
	0.4	51	91	185	221		226
	0.6	55	120	210	232		
	1.0	48	129	210	224		
B. Commercial dried yeast	0.3		21			218	233
	0.6		15		167	228	235
	1.0		12		177	231	238

to that of fresh yeast, and that in every case an induction period of many hours elapsed before active fermentation occurred. With the combination of fresh and dried yeast, however, (see Preparation of A + D) fermentation started without delay and was nearly complete in 2 hours.

Effect of Varying Quantity of Activator—The effect upon rate of maltose fermentation of varying quantity of activator was noted in the case of the commercial zymin and of dried bakers' compressed yeast, these preparations, respectively, being superimposed in varying amounts upon mixtures containing 0.3 gm. of fresh yeast and 0.3 gm. of maltose. Comparative fermentation rates are indicated in Table II.

It is apparent from the values given in Table II that for Activator A, within wide limits, the stimulating effect on fermentation is not proportional to the amount used, although the larger quantities do significantly increase the initial fermentation rate. In the case of Activator B, which had a much longer induction period than A, quantity variations from 0.3 to 1 gm. were without significant effect.

Properties and Identities of Maltose Fermentation Activators in Dried Yeast Preparations—From the foregoing experiments the obvious conclusion is that the drying of yeast induces the formation of one or more factors that stimulate to a remarkable degree the maltose-fermenting capabilities of fresh bakers' yeast. The likelihood that there is more than one of these factors has been suggested in the discussion of data presented in Fig. 3.

It has been indicated that the maltose fermentation activator found in wheat flour might be identical with von Euler's Factor Z, and the same should reasonably be assumed for at least one of the dried yeast factors. Assuming two types of activators in dried yeast preparations, one type conspicuous for its ability to eliminate the induction period, experiments were designed to disclose, if possible, any distinctive differences in the properties of the two types.

Stability toward heat (boiling in water) and dialyzability were properties suggestive of the likelihood that the wheat flour activator is identical with Factor Z, but with dried yeast preparations, however, boiling in water—in fact, moist heat at a temperature far below 100°—rapidly destroyed much of their effectiveness as accelerators of *maltose* fermentation. Especially prominent was the destructive effect upon the particular factor that is responsible for the elimination of the induction period. This seems to show that dried yeast contains an active maltose-fermenting accelerator that is distinct from Factor Z, since the latter is known to be resistant to boiling water temperature. A means for the identification and measurement of Factor Z is to observe the extent to which the substance in question, after boiling, is capable of accelerating the fermentation of glucose or sucrose, by fresh yeast. It was therefore considered of interest to compare the accelerating effects of the dried yeast activator, both before and after boiling, on sucrose and maltose fermentation, respectively. Typical data obtained from this series of studies are given in Table III.

Both dried yeast preparations showed marked ability to accelerate the fermentation of sucrose by fresh yeast, but this stimulation of sucrose fermentation is by no means as striking as the effect upon maltose fermentation.

Boiling treatment of the dried yeast preparations strongly inhibited their power to accelerate maltose fermentation, this effect being far more drastic for the zymin than for the dried compressed yeast. It is highly significant, however, that the

TABLE III

Acceleration of Maltose and Sucrose Fermentation by Boiled and Unboiled Dried Yeast Preparations

A. Fresh yeast alone—no activator
B. "   "   + dried compressed yeast, unboiled
B_1. "   "   + "   "   "   boiled 10 min.
C. "   "   + commercial zymin, unboiled
C_1. "   "   + "   "   boiled 10 min.

0.3 gm. of fresh yeast, 0.3 gm. of activator, and 0.3 gm. of sugar were used
CO_2 pressures are measured in mm. of Hg.

Prepara- tion	Substrate	CO_2 pressures at successive time intervals					
		$\frac{1}{2}$ hr.	1 hr.	2 hrs.	3 hrs.	5 hrs.	7 hrs.
A	Maltose	5	7	9	13	20	25
"	Sucrose	23	60	135	183	208	213
B	Maltose	33	68	173	220	228	
"	Sucrose	51	117	223	249	252	
C	Maltose	43	91	179	223	227	
"	Sucrose	44	113	228	242		
B_1	Maltose	6	8	37	106	227	
C_1	"	2	3	7	18	80	
B_1	Sucrose	49	114	219	236		
C_1	"	48	124	228	245		

boiling treatment did *not* appreciably impair the ability of either of the activator substances to accelerate *sucrose* fermentation. This, seemingly, is conclusive evidence that, in addition to von Euler's Factor Z, the dried yeast preparations contained a catalyst that is specific for maltose fermentation. The latter, in contrast to Factor Z, is destroyed by boiling. It may, for convenience, be tentatively designated as Factor M.

That Factor M is not a definite and exclusive chemical entity, having constant properties irrespective of origin or source, is

evidenced by the fact that the activator found in malted wheat flour is far more stable toward heat, as well as toward other treatments, than the one present in certain dried yeast preparations. The latter is injured not only by boiling, but also by exposure to alcohol. It is only partially dialyzable, and only partially extractable with water. When a portion of the commercial zymin was electrodialyzed, much of the Factor M was destroyed, although the liquid in the cathode compartment was found to contain considerable activity.

It is, of course, possible that the presence of certain unrecognized *inhibitors* may have played a prominent part in some of the phenomena herein discussed, and that this has led to some misinterpretation. Several attempts to inquire into this possibility gave negative results, however. Control of hydrogen ion concentration did not appear to be an issue of serious consequence.

Only a few pure chemical substances were investigated as to their possible stimulating effects upon maltose fermentation by fresh yeast. These included varying quantities of cysteine, cystine, glutathione, and monobasic ammonium phosphate, respectively. None gave significantly positive results.

Effect of Dried Yeast Activator on Completeness of Sugar Fermentation—The calculated theoretical yield of CO_2 from a specified quantity of sugar is usually not realized in ordinary yeast fermentation. In experiments with pure glucose and sucrose it was consistently observed that the addition of activator to the fresh yeast and sugar accomplished more than a mere acceleration of fermentation rate. There was also a very substantial reduction of the "fermentation deficit," *i.e.* the difference between theoretical and actual yields of CO_2. For sucrose our calculations—based upon volume and temperature considerations—indicated that 0.3 gm. should give a pressure of about 282 mm. of mercury, assuming *complete* conversion to CO_2 and alcohol by fermentation. With 0.3 gm. of fresh compressed yeast, together with the activator supplement, final pressures averaging 253 mm. were reached, indicating approximately 90 per cent of the maximum theoretical yield. This value was consistently obtained, within a small factor of error, with amounts of dried yeast preparations varying from 0.2 to 2.0 gm. With 0.3 gm. of fresh yeast *alone*, and the same quantity of sucrose, closely agreeing values averaging 214

mm. were obtained, indicating that alcoholic fermentation was only 76 per cent complete. From these values of 90 and 76 per cent it may be calculated that without the activator the fermentation of sucrose was only 85 per cent as efficient as with the activator. It must be presumed from the data given in Table III that for sucrose fermentation Factor Z, not Factor M, is the activator responsible for the increased total CO_2 production, because this particular potentiality was not impaired by the boiling treatment which was highly destructive to Factor M.

Convenient Method for Preparation and Use of Sugar Fermentation Activators—Several readily available sources of Factor M have been discussed. It is perhaps safe to expect that all dried or autolyzed yeast preparations containing Factor M will also contain Z, but the reverse of this is certainly not true, as in the case of boiled zymin. Accordingly, since Factor Z of yeast is much more stable than Factor M, the production and preservation of Factor M is the critical feature of any procedure in which both types of biocatalysts are desired.

To obtain highly active preparations of Factor M from fresh yeast, the yeast must be dried but not overheated while moist. Any method in which the fresh yeast is autolyzed, *i.e.* putting it on a steam bath or in a hot air oven, must be avoided. If dried in the vacuum oven, fairly high temperatures may be used. A highly satisfactory and simple procedure is to crumble up a quantity of compressed filler-free yeast, spread it over a flat surface, and use an overhead heater, with a current of air supplied by a fan. If stirred occasionally, complete drying is accomplished in 3 or 4 hours. This dried yeast need not be finely ground, because it readily disintegrates in water suspension.

Dried yeast thus prepared contains an appreciable amount of fermentable substance, which may either be fermented out or corrected for by blank determinations. If made into a paste or batter with water and allowed to stand a few hours, the small quantity of fermentable substance is disposed of and the yeast is redried by the method already described. When maximum accuracy is desired, it is advisable to run blank determinations with fresh yeast and activator, alone, with each series of tests.

Detection of Activators in Biological Material—The simple and obvious method for identification of fermentation activators in

biological substance is to ferment, under comparable and controlled conditions, two samples of the material, one in the presence and the other in the absence of activator substance, *i.e.* the dried yeast preparation.   If the material under consideration is deficient in activator, we can expect the added dried yeast preparation significantly to increase both the *speed* and *completeness* of fermentation, the degree of response indicating the extent of the deficiency in the material itself.   If, on the other hand, the material itself contains activator, this should be indicated by either a negative or an insignificant response in the test in which the dried yeast activator preparation was added.

TABLE IV

Comparative Effects of Activator on Fermentation of Different Types of Biological Material

0.5 gm. of yeast was used.

Sample	Weight of sample	Activator	CO_2 pressures at successive time intervals					
			1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.
	gm.	*gm.*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*
Carrots.............	1	0	168	278	288	291	291	
"	1	0.5	166	286	295	295	296	
Molasses	5	0	97	140	151	160	165	168
"	5	0 5	135	176	181	189	192	192

As illustrative of two types of biological products, one containing fermentation activators while the other is lacking or deficient in this respect, we may consider carrots on the one hand and molasses on the other.   Under comparable conditions, when tested both with and without the dried yeast supplement, fermentation tests on these two materials gave, respectively, the results typified in Table IV.

In Table IV it is seen that in the case of the carrot sample the supplementary effect of added activator on both rate and completeness of fermentation was insignificant as compared with the effect upon the molasses.   This is interpreted to mean that the carrot material, itself, contained sufficient activator to render unnecessary any addition of the dried yeast preparation.

On the other hand the molasses was highly deficient in this

respect, for without added activator its rate of fermentation was much slower, and the final yield of carbon dioxide was about 87 per cent of the yield when supplemented by the dried yeast activator.   This value of 87 per cent assumes added significance when it is recalled that the final carbon dioxide yield for pure sucrose *without* added activator was approximately 85 per cent of the yield obtained in the presence of the activator substance.

Findings of this character indicate the importance of insuring the presence of an adequate amount of these biocatalytic factors in technological studies involving the quantitative estimation of sugars by fermentation methods.   A knowledge and appreciation of the properties, use, and control of these biocatalysts should prove of value in the further improvement and establishment of such fermentation methods.   From the standpoint of convenience and simplicity, alone, the tremendous advantages of the type of fermentation procedure used in these studies justifies every effort to establish the conditions and limitations under which such methods are applicable and trustworthy.   Suggested possibilities for the use of this type of method in studying amylase activity appear in papers, respectively, by Schultz and Landis (8), Schultz and Kirby (9), Sandstedt and Blish (5), and Sandstedt, Blish, Mecham, and Bode (10).   Further studies are in progress.

SUMMARY

Fresh bakers' compressed yeast is usually strikingly deficient in its ability to ferment pure maltose, alone, as compared with maltose in the presence of other biological substances.

Flour (especially from malted wheat) and dried yeast preparations, respectively, contain accelerators that are specific for maltose fermentation and that are distinctive for the ability to (1) eliminate or greatly shorten the induction period, (2) increase fermentation rate, and (3) reduce the "fermentation deficit."

The maltose fermentation accelerator (Factor M) in dried yeast preparations is sharply distinguishable from Factor Z of von Euler by its great instability toward heat and toward alcohol.

An understanding of the properties and the capabilities of fermentation activators is highly important in the development and use of fermentation methods for the quantitative estimation of sugars, and for the study of phenomena associated with amylase

activity. The manometric yeast fermentation technique affords an extraordinarily simple and convenient procedure for technological studies of this character.

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ON PROTEOLYTIC ENZYMES

XIV. ON THE GENERAL NATURE OF THE ENZYMATIC DEGRADATION OF PROTEINS

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The general conception has been that the enzymatic degradation of proteins proceeds in two distinct stages; *i.e.*, the proteinases cleave the true protein into polypeptides and from this point the polypeptidases and dipeptidase complete the degradation to amino acids. The existence of enzymes that act directly on proteins and break them down to amino acids has been explicitly denied (1–3). The reports describing the formation of amino acids in pepsin or papain digests of proteins have been rejected on the grounds of errors in the methods. For example, in discussing the investigation of Calvery (4) it is assumed that the tyrosine liberated during the peptic digestion of egg albumin was not formed by the action of the enzyme but by the acid in the digestion mixture (1). It appears that these objections cannot be valid in all cases,[1] as Annetts (6) has shown, with the aid of the ultracentrifuge, that amino acids and lower peptides are formed when egg albumin is digested by papain; and Calvery and his coworkers have offered additional evidence (7–9) supporting his earlier conclusions.

In previous studies from this laboratory (10–12) it has been shown that amino acids are liberated from synthetic peptides by the action of papain. Therefore it was expected that an exhaustive digestion of a protein would yield considerable amounts of free amino acids. It did not seem superfluous to conduct a

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[1] In several instances there are ample grounds for valid objections; see, for example, Emmerling (5).

digestion of this kind and to isolate, in a pure condition, at least a part of the amino acids so formed. Furthermore, it appeared desirable to determine whether both of the partial enzymes of papain were capable of liberating amino acids from proteins. Cattle fibrin was selected for digestion and the amino acids that were isolated are given in Table I. The tyrosine separated directly from the digest and the other amino acids were obtained by extraction with butyl alcohol, a method which Dakin (15) has recommended for the investigation of enzymatic digests.

Practically all of the tyrosine and considerable amounts of the phenylalanine, tryptophane, leucine, and isoleucine present in fibrin were liberated on digestion with papain. Free proline was

TABLE I

Amino Acids Isolated from Fibrin-Papain-HCN Digest

Amino acid	Amount	
	Isolated	In fibrin*
	per cent	*per cent*
Tyrosine........	3.2	3.5
Tryptophane..........	1.6	5.0
"Leucines".....	6.7	15.0
Leucine.............................	3.1	
Isoleucine...........................	0.7	
Phenylalanine.........	0.3	2.5

* See Abderhalden and Voitinovici (13) and Bergmann and Niemann (14).

not found in the papain-fibrin digest as was anticipated from the earlier studies on synthetic papain substrates undertaken in this laboratory.

In addition to the above experiment, fibrin was digested with papain in the presence of phenylhydrazine. Under these conditions Papain I is inactive and Papain II is responsible for the digestion (16, 17). In this case, 1.1 per cent of tyrosine were isolated, which is one-third of the amount obtained through acid hydrolysis or through the combined action of Papain I and Papain II. Therefore it is evident that both Papain I and Papain II can liberate tyrosine from the protein molecule.

It has been pointed out in previous researches of this laboratory that proteinases hydrolyze peptides or peptide derivatives of

low molecular weight, and that simple substrates have been found for papain, liver cathepsin, bromelin, chymotrypsin, and heterotrypsin.[2] Therefore, the conception that proteinases are limited to the splitting of substrates of high molecular weight and that amino acids and lower peptides are the result of the secondary action of enzymes other than proteinases does not appear to be valid.

As proteins of high molecular weight contain only a vanishingly small number of free α-amino and α-carboxyl groups, the first step in their enzymatic degradation can be accomplished only by enzymes which do not require a free α-amino or α-carboxyl group; *i.e.*, the proteinases or endopeptidases. The extent of the splitting of proteins by proteinases is determined by the structure of the protein and by the specificity of the proteinase. In many cases the degradation proceeds with the formation of large amounts of amino acids. However, the specificity of the proteinases is such that they cannot degrade a protein completely to amino acids. To attain this state, the action of the proteinase must be supplemented by the action of exopeptidases, *e.g.* carboxypolypeptidase, dipeptidase, etc.

<center>EXPERIMENTAL</center>

Preparation of Digest—1172 gm. of cattle fibrin, 1635 cc. of an aqueous solution containing 27 gm. of papain (19) and 6.5 gm. of HCN, 1635 cc. of 0.2 M disodium phosphate, 6500 cc. of water, and 20 cc. of toluene were incubated at 37° for 20 days.[3] The digest (pH 6.4) was heated to 80° and filtered, and the residue was exhaustively extracted with hot water. The concentrated washings were added to the original filtrate and the solution was made up to 14 liters.

Isolation of l-Tyrosine—After the digest stood at 6° for 3 weeks, 29.2 gm. of *l*-tyrosine separated from it. The crude product was recrystallized from water.

$$C_9H_{11}O_3N. \quad \text{Calculated, N 7.7; found, N 7.7}$$
$$[\alpha]_D^{25} = -15.5° \ (c = 1.6\% \text{ in } 10\% \text{ HCl})$$

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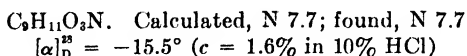
[2] This enzyme, which splits hippuryllysineamide, is described elsewhere by Bergmann and Fruton (18).

[3] Approximately 64 per cent of the peptide bonds were hydrolyzed under these conditions.

When the minimum solubility of tyrosine in 14 liters of solution is accounted for, the yield is increased by 7.0 gm.[4]   Thus the total yield was 36.2 gm. or 3.1 per cent of the protein.

Butyl Alcohol Extraction—13.4 liters of digest were shaken twice with an equal volume of *n*-butanol.   The alcoholic phases were concentrated to 400 cc. and 73.8 gm. of solids were recovered. The aqueous phase was then concentrated below 35° to 3.8 liters and extracted 5 times with equal volumes of butanol.   82.3 gm. of solids were recovered from the alcoholic phases.   Thus the total yield was 156.1 gm.

Isolation of l-Leucine—125 gm. of the above solids were dissolved in 1 liter of water.   After the mixture stood overnight at 6°, 6.8 gm. precipitated.   4.3 gm. of this product were dissolved in 80 cc. of water and treated with 10 gm. of potassium cyanate (20).   After acidification, the hydantoic acid was collected (4.5 gm.) and recrystallized from water.   The acid melted at 211–212° with decomposition.

$C_7H_{14}O_3N_2$.   Calculated, N 16.1; found, N 15.9   .

The hydantoic acid was converted into the hydantoin and after recrystallization from water the substance melted at 214.0–214.5°.   A mixed melting point with an authentic specimen of isobutylhydantoin showed no depression.

$C_7H_{12}O_2N_2$.   Calculated, N 17.9; found, N 18.0

4.3 gm. of pure *l*-leucine yielded, under the same conditions, 5.0 gm. of the hydantoic acid.   Therefore approximately 90 per cent of the above precipitate was *l*-leucine.

Isolation of l-Tryptophane—The aqueous solution remaining after the crystallization of the crude leucine was acidified with sulfuric acid and the tryptophane was precipitated in the usual manner with Hopkins-Cole reagent.   When the precipitate was decomposed and the solution was concentrated to 75 cc., 12.0 gm. of crude tryptophane were obtained.   After one recrystallization from aqueous ethanol the substance possessed the correct constants (21).

$C_{11}H_{12}O_2N_2$.   Calculated, N 13.7; found, N 13.8
$[\alpha]_D^{25} = +2.8°$ ($c = 2.1\%$ in 10% HCl)

[4] During subsequent operations 5.0 gm. were actually isolated.

When the tryptophane mother liquor was treated with formaldehyde and sulfuric acid (22), 2.3 gm. of 3,4,5,6-tetrahydro-4-carboline-5-carboxylic acid were obtained. After recrystallization from 50 per cent ethanol containing a trace of ammonia, the substance melted at 310° with decomposition.

$$C_{12}H_{12}O_2N_2. \quad \text{Calculated, N 13.0; found, N 13.2}$$

Isolation of d-Isoleucine—The filtrate from the tryptophane precipitation was freed of inorganic ions and concentrated to 100 cc. 38.2 gm. of crude "leucine" were isolated from this concentrate. 35.0 gm. of this product (N 10.7) were dissolved in 1.5 liters of water and treated with 50 gm. of cupric hydroxide. After the mixture stood overnight at 25°, the precipitate was collected, washed, suspended in water, and decomposed with hydrogen sulfide. The solution was concentrated to a small volume, and with the aid of ethanol 15.0 gm. of *l*-leucine were obtained.

$$C_6H_{13}O_2N. \quad \text{Calculated, N 10.7; found, N 10.8}$$
$$[\alpha]_D^{25} = +17.0° \ (c = 2.1\% \text{ in } 10\% \text{ HCl})$$

The α-naphthyl isocyanate compound melted with effervescence at 158–159°.

$$C_{17}H_{20}N_2O_3. \quad \text{Calculated, N 9.3; found, N 9.6}$$

The solution containing the water-soluble copper salts was reduced to 500 cc. and filtered, and the filtrate was freed of copper. The solution was concentrated to 50 cc., and with the aid of ethanol 5.5 gm. of crude isoleucine were isolated. 4.5 gm. of this product were treated with α-naphthyl isocyanate and after recrystallization from 50 per cent ethanol the derivative melted with decomposition at 178–179°.

$$C_{17}H_{20}N_2O_3. \quad \text{Calculated.} \quad \text{C 68.0, H 6.7, N 9.3}$$
$$\text{Found.} \qquad \text{" 67.6, " 6.9, " 9.6}$$
$$[\alpha]_D^{25} = +29.0° \ (c = 9.5\% \text{ in ethanol})$$

A melting point of 178° and a specific rotation of 29.5° have been reported for the α-naphthyl isocyanate compound of *d*-isoleucine (23).

Isolation of l-Phenylalanine—The mother liquor from the crude

leucine separation was treated with an excess of cupric hydroxide. The precipitate was collected, suspended in water, and decomposed with hydrogen sulfide, and the solution was concentrated to 200 cc. 100 cc. of this solution were treated with 4 gm. of picrolonic acid and the precipitate was discarded. The filtrate was treated with an additional 4 gm. of picrolonic acid and the crystalline precipitate was collected (3.3 gm., N 15.9). A sample recrystallized twice from hot water sintered above 100° and melted with decomposition at 200°.

$C_{19}H_{19}O_7N_5 \cdot H_2O$. Calculated. C 51.0, H 4.7, N 15.7, H_2O 4.0
Found. " 50.9, " 4.9, " 15.9, " 4.1

When the derivative was treated with chromic acid, the characteristic odor of phenylacetaldehyde was recognized. The other 100 cc. of the above solution were treated with potassium cyanate, and 6.5 gm. of a crude hydantoic acid were isolated. Alternate recrystallizations from hot water and alkali raised the melting point to 205–206°, thereby identifying the substance as N-carbamylleucine.

$C_7H_{14}O_3N_2$. Calculated, N 16.1; found, N 16.0

Examination of Fraction Soluble in Butanol—The butanol mother liquors were combined and the solvent was removed *in vacuo*. The residue (30 gm.) was taken up in ethanol and filtered, and the filtrate was freed of ethanol. The residue was taken up in 100 cc. of water and portions of this solution were treated with rhodanilic acid (24), but in no case were the characteristic crystals of proline rhodanilate observed. When *l*-proline was added to the solution under examination, the amino acid was readily recovered, thereby indicating the absence of free proline in the enzymic digest.

Additional Isolation Experiments—The aqueous phase remaining after the butanol extractions was shaken out three times with 4 liters of *n*-propanol. The alcohol phases were combined, the solvent was removed, and the residue was taken up in cold 5 per cent sulfuric acid. An excess of Hopkins-Cole reagent was added, the precipitate was removed, and the filtrate was freed of inorganic ions. 13.1 gm. of solids were isolated from this

solution. Examination of this fraction indicated that the substance was a mixture of isomeric leucines.

$C_6H_{13}O_2N$. Calculated, N 10.7; found, N 10.6

The aqueous phase remaining after the propanol extractions was filtered and yielded 5.0 gm. of tyrosine.

$C_9H_{11}O_3N$. Calculated, N 7.7; found, N 7.7

Papain-HCN-Phenylhydrazine Digestion—246 gm. of cattle fibrin, 375 cc. of an aqueous solution containing 6.25 gm. of papain, 1.5 gm. of HCN, and 7.3 gm. of phenylhydrazine,[5] 375 cc. of 0.2 M disodium phosphate, 1750 cc. of water, and 10 cc. of toluene were incubated at 37° for 20 days. A control experiment without phenylhydrazine was conducted at the same time. At the end of 20 days and after correction was made for the enzyme blanks, the papain-HCN digest contained 6.35 mg. of amino N per cc., and the papain-HCN-phenylhydrazine digest 5.91 mg. of amino N per cc. Sufficient benzaldehyde was added to the digest containing the phenylhydrazine to react with the base, and the two solutions were heated just to boiling. The insoluble matter was removed and washed with hot water. The filtrate and washings were combined and the solutions concentrated *in vacuo* to 1 liter. After the mixture stood at 6° for 3 months, the crude tyrosine was collected. 7.3 gm. of tyrosine were obtained from the papain-HCN digest, but only 2.3 gm. were recovered from the papain-HCN-phenylhydrazine hydrolysate. Thus, in the case of the papain-HCN digestion, after correction was made for the minimum solubility of tyrosine in the solution, the yield was 7.8 gm. or 3.2 per cent of the protein.

$C_9H_{11}O_3N$. Calculated, N 7.7; found, N 7.7

The corrected yield in the case of the digestion conducted in the presence of phenylhydrazine was 2.8 gm. or 1.1 per cent of the protein.

$C_9H_{11}O_3N$. Calculated, N 7.7; found, N 7.8

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[5] This solution was incubated at 37° for 2 hours before it was added to the other components.

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β-ESTRADIOL

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Several years ago Schwenk and Hildebrandt (1) reported in a preliminary publication the preparation of two isomeric forms of estradiol, by catalytic reduction of estrone, differing from each other by their melting points (170° and 204°). The lower melting isomer was found to be more active in the Allen-Doisy test than the higher melting compound, and has attained physiological importance since MacCorquodale, Thayer, and Doisy (2) isolated it from liquor folliculi. This substance is the most potent estrogenic compound known as yet and in all probability represents the true follicular hormone of the ovary. It has also been shown to be present in the urine of pregnant mares by Wintersteiner, Schwenk, and Whitman (3).

Later, other workers (2, 4–7) reduced the keto group of estrone by catalytic hydrogenation or with sodium in alcohol, but obtained only the lower melting diol. It was suggested by Girard and coworkers that the high melting isomer was actually derived not from estrone but from an accompanying impurity.

In the following we describe in more detail a new method of reduction and the separation and properties of the high melting isomer. To facilitate discussion we use tentatively the terms α-estradiol for the low melting, and β-estradiol for the high melting isomer. According to their mode of formation the two isomers can differ from each other only by the stereochemical configuration of carbon atom 17. They therefore may be regarded as epimers.

The reducing agent is a nickel-aluminum alloy,[1] which is al-

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[1] Raney's nickel-aluminum, obtained from the Gilman Paint and Varnish Company, Chattanooga.

lowed to react on an alkaline solution of estrone at 80–90°. The crude reaction product consists of a mixture containing preponderantly the α epimer. Its specific rotation indicates a content of no more than 10 to 20 per cent of the β compound. By repeated recrystallization α-estradiol is easily obtained, while the combined mother liquors yield products melting between 190–206°. The separation of these mixtures by fractional crystallization is rather laborious, but fractions melting from 200–210° finally accumulate, and from these the β compound can be obtained without further difficulty. A far more convenient method for the separation of these mixtures has recently been found by one of us (Wintersteiner (8)). It is based on the fact that the α isomer is capable of forming a sparingly soluble digitonide in 80 per cent alcohol solution, while the β isomer is not precipitated by digitonin under these conditions. The implications of this finding have been discussed in a separate communication (8). The pure α isomer can be recovered from the digitonide by the pyridine method. The filtrate from the digitonide, after removal of the digitonin, easily yields the pure β isomer after several recrystallizations.

β-Estradiol crystallizes from dilute ethyl alcohol in needles which melt with slight decomposition at 220–223° (corrected). A 3-monobenzoate, m.p. 156–157° (corrected), and a diacetate, m.p. 139–141.5° (corrected), have been prepared.

Schwenk and Hildebrandt found that α-estradiol was more potent physiologically than β-estradiol. Since their preparations, especially that of the β isomer, were not homogeneous, as is indicated by the lower melting points, the bioassays have been repeated with the pure isomers now available.

The Allen-Doisy method applied to the ovariectomized rat was used for standardization. The material to be tested was injected in sesame oil. Pure estrone was repeatedly assayed by this method and found to contain 1,000,000 rat units per gm. Our rat unit is therefore 10 times the international unit (0.1 microgram of estrone).

The figures obtained with the diols and their benzoates are given in Table I. Our present measurements indicate that α-estradiol is about 12 times as potent as estrone, while the ratio previously determined was only 6:1. MacCorquodale, Thayer,

and Doisy give a unitage of 16,600,000 international units, measured by the modified Allen-Doisy procedure (injection of aqueous solutions), which corresponds to a ratio of 1.6:1. David, de Jongh, and Laqueur (6) find an activity ratio of 3:1 under conditions more comparable with ours (injection of oil solutions, undivided dose). These discrepancies in the results of various investigators can probably be ascribed to differences in the assay technique. The variability of assay results obtained under different conditions has been carefully studied and discussed by the Dutch investigators.

TABLE I

Properties of α, β-Estradiols and Their Derivatives

	M.p.	$[\alpha]_D$	Physiological activity
	°C.	degrees	rat units per gm.
α-Estradiol	178	+81	12,000,000
β-Estradiol	223	+54	300,000
α-Benzoate	195		6,000,000
β-Benzoate	157		150,000
α-Diacetate	127		
β-Diacetate	141.5		
Estrone	258–260	+165	1,000,000

The benzoate of α-estradiol is, according to our assays, 6 times as strong as estrone, which is not far removed from the ratio found by David *et al.* (3 to 4:1).

β-Estradiol, on the other hand, contains only about 30 per cent of the activity of estrone according to our present assay. The high ratio (4:1) which was found originally by Schwenk and Hildebrandt with their impure preparation was undoubtedly due to contamination with the highly active α isomer.[2]

[1] A substance melting at 224° and apparently identical with β-estradiol has recently been isolated from the urine of pregnant mares by Hirschmann and Wintersteiner (unpublished experiments). This preparation was assayed simultaneously with the substance described in this paper. However, the urinary preparation was found to be even less active (50,000 to 100,000 rat units per gm.) than the β-estradiol obtained by reduction. These figures are preliminary, but, if substantiated by further assays, they would indicate that our preparation of β-estradiol obtained by reduction contained still a small amount (2 per cent or less) of the α isomer.

Benzoylation of the hydroxyl at carbon atom 3 decreases the activity to half of that of the free diol, as in the case of the α isomer.

A similar situation in regard to physiological activity obtains with the C 17 epimers of testosterone (9). The isomer occurring in the testes, named *trans*-testosterone by Ruzicka and collaborators, on the basis of certain chemical evidence relating the configuration of the C 17 hydroxyl to the methyl group on carbon atom 13, is the most potent androgenic compound known. On the other hand, the *cis* epimer, derived from a by-product in the reduction of Δ_5-androstene-3-ol-17-one-3-acetate, shows only little activity in the capon test and is entirely inactive in the seminal vesicle and prostate test in the castrated rat. The biological criteria as well as the relation of the melting points and specific rotations of the two epimeric pairs would therefore seem to suggest an identical configuration on carbon atom 17 in α-estradiol and *trans*-testosterone on the one hand, and of β-estradiol and *cis*-testosterone on the other hand.

EXPERIMENTAL

Reduction of Estrone—A technical preparation of estrone, isolated from the urine of pregnant mares, was purified by the quinoline method of Butenandt and Westphal (10) and recrystallized twice from acetone. The final product (m.p. 258–260° (uncorrected); $[\alpha]_D = +165°$) was used for the reduction experiment.

A solution of 1 gm. of estrone in 1300 cc. of 10 per cent aqueous potassium hydroxide was heated to 80–90°. 18 gm. of Raney nickel were then introduced in small portions with vigorous stirring, care being taken that the evolution of hydrogen had ceased before each new addition. The hot reaction mixture was filtered quickly and the residue on the filter washed thoroughly with water in such a manner that it was always covered with fluid. This precaution is necessary as the nickel residue will ignite if allowed to become dry. The filtrate, while still hot, was acidified to Congo red with hydrochloric acid, and the precipitate filtered after cooling. Precipitation in the cold yielded a difficultly filtrable product.

Separation of Isomers by Fractional Crystallization—The crude

reaction product is a white powder melting at 168–180°. Two recrystallizations from acetone gave fairly pure α-estradiol (m.p. 172–174°) in 60 to 80 per cent yield. On further recrystallization from 80 per cent alcohol the pure compound, which melts at 176–178° (corrected), is obtained in the form of short prisms; $[\alpha]_D = +81°$ (1 per cent in alcohol). The monobenzoate and the diacetate which we prepared melted at 194–195° and 126–127° respectively, in accordance with the data given by David, de Jongh, and Laqueur (6).

In the acetone mother liquors crystalline material remains which softens at 170° and melts at 190–204°. Further fractionation with 80 per cent alcohol yields more of the α isomer, and, in the more soluble fractions, needle-shaped crystals which melt at 200–206° and probably represent mixed crystals of both isomers. The further fractionation of these mixtures is laborious, but finally fractions melting around 210° can be obtained, which on further recrystallization yield the pure β isomer without difficulty.

Thus 175 mg. of a product melting at 190–204° gave 50 mg. of the α isomer melting at 170–174° and 27 mg. of the β isomer melting at 218–223°.

Digitonin Procedure—55 mg. of material obtained from the intermediate fractions of the above fractional crystallizations were dissolved in 3 cc. of 80 per cent alcohol. To this solution were added 10 cc. of a solution of 200 mg. of digitonin in 80 per cent alcohol. After 24 hours standing at room temperature the crystalline digitonide was filtered off and washed with small portions of 80 per cent alcohol. After drying, the crystals (93 mg.) were dissolved in 2 cc. of dry pyridine, and 7 cc. of anhydrous ether were gradually added. The precipitated digitonin was removed by centrifuging. The supernatant liquid was washed with dilute sulfuric acid and water, and brought to dryness. The residue (22 mg.) on recrystallization gave 17.7 mg. of α-estradiol melting at 176–178° (corrected). Further recrystallization did not change the melting point.

The filtrate from the digitonide was brought to dryness *in vacuo* and the residue extracted with dry ether. The ether-soluble material (34 mg.) on recrystallization yielded 22 mg. of crystals melting at 204–214°. These were twice recrystallized and then consisted of the pure β isomer.

β-Estradiol crystallizes from dilute alcohol in long, shiny needles melting at 220–223° with slight decomposition; $[\alpha]_D^{24} = +53.8°$ (0.7 per cent in dioxane). The ultraviolet absorption spectrum is identical with that of estrone. In concentrated sulfuric acid the compound gives an intense blue fluorescence, but with a slight greenish tint, which is absent with the *α* isomer. Coupling with *p*-nitrodiazobenzene in alkaline solution produces a yellow dye of the same shade as that given by the *α* isomer. The preparation crystallized from dilute alcohol contained solvent of crystallization which could not be completely removed at 80° even in a high vacuum. A similar behavior has been noted with *α*-estradiol (6).

Analysis—(Dried at room temperature)
$C_{18}H_{24}O_2 \cdot \frac{1}{2}H_2O$. Calculated. C 76.81, H 8.96
Found. " 77.11, " 8.81
(Dried at 110° at 2 mm.)
$C_{18}H_{24}O_2$. Calculated. C 79.35, H 8.89
Found. " 79.33, " 8.83

*β-Estradiol-3-Benzoate—*23 mg. of *β*-estradiol were dissolved with warming in 5 cc. of 5 per cent sodium hydroxide solution. After cooling to room temperature an excess of freshly distilled benzoyl chloride was added drop by drop with vigorous shaking. The precipitated benzoate, after washing with sodium hydroxide and water, weighed 29.8 mg. It was recrystallized twice from 90 per cent alcohol; the platelets melted at 156–157°.

Analysis—$C_{25}H_{28}O_3$. Calculated. C 79.74, H 7.50
Found. " 79.71, " 7.46

*β-Estradiol Diacetate—*8.4 mg. of *β*-estradiol were boiled for $1\frac{1}{2}$ hours with pure acetic anhydride. The reagent was distilled off *in vacuo* and the residue recrystallized twice from 80 per cent alcohol; 6.3 mg. of prisms with oblique ends, m.p. 139–141.5° (corrected), were obtained.

Analysis—$C_{22}H_{28}O_4$. Calculated. C 74.12, H 7.92
Found. " 74.10, " 7.80

*Bioassay—*The bioassays were conducted in the laboratory of Dr. Charles Mazer (Philadelphia), to whom we wish to extend our thanks for his kind cooperation. Ovariectomized rats, kept under

standard conditions, were injected three times in the course of a day with a solution in sesame oil of the substance to be tested. A total of three injections was given.   50 rats in groups of ten were employed for each assay, each group receiving a different dose.   The five group dosages were graded in such a way that the highest dose was 3 times as great as the lowest.   The total dose per rat producing full estrus in seven out of the ten rats was taken as the equivalent of 1 rat unit.

SUMMARY

Reduction of estrone with nickel-aluminum yields two isomeric diols, which differ from each other by the configuration of carbon atom 17. α-Estradiol, which is identical with the dihydrotheelin isolated by MacCorquodale, Thayer, and Doisy from follicular fluid, is formed predominantly. The properties of the other epimer, β-estradiol, which is much less active physiologically than α-estradiol, are described.

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